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DNA SEQUENCE ENCODING THE TUMOR SUPPRESSOR ING1

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No. 08/569,721 which is incorporated in its entirety by reference herein.

5 FIELD OF THE INVENTION

This invention relates to a novel tumor suppressor gene, ING1, to methods for making and using this and related tumor suppressor genes and proteins and peptides, and to nucleic acids encoding this and related tumor suppressor proteins and peptides.

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The disclosure of the above publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if the language of each individual publication, patent and patent application were specifically and individually included herein.

BACKGROUND OF THE INVENTION

Many cancers originate and progress by accumulating mutations in one or more genes. Such mutations which result in cancer formation can be in 30 proto-oncogenes or in tumor suppressor genes. Mutations in tumor suppressor genes result in loss of function, and therefore act in a recessive fashion to native

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genes. Oncogenes, in contrast, act in dominant fashion to native alleles and, therefore, are not usually inherited through the germ lines. The tumor suppressor genes, however, are found in inherited predispositions to cancer and are inherited as a dominant predisposition because of the high frequency of a 5 second genetic event such as reduction in homozygosity[1].

Several tumor suppressor genes have been identified. Examples include the Rb gene, which is involved in retinoblastoma and osteosarcoma; p53, which is involved in osteosarcoma and adrenocortical, breast and brain cancers; WT-1, which is involved in Wilms' tumor, nephroblastoma and neurofibromatosis; 10 adenomatous polyposis coli (APC), which is involved in adenomatous polyposis; and deleted colorectal cancer (DCC), which is involved with a somatic mutation in the colon.

The negative regulation of cell growth is effected by tumor suppressor proteins that regulate the cell cycle by different mechanisms[2]. The gene 15 cloned and sequenced as described herein, ING1 (formerly called p33^{IG1}), represents a new tumor suppressor gene which is expressed in normal mammary epithelial cells, but expressed only at lower levels in several cancerous mammary epithelial cell lines and is not expressed in many primary brain tumors.

20 Known applications of sequenced genes include use of the DNA sequence (or analogs thereof) or of RNA or amino acid sequences derived from these DNA sequences for diagnosis or treatment of the corresponding disease. Accordingly, the gene ING1 (previously designated p33^{IG1}) is useful for the diagnosis and treatment of breast and brain cancers among others.

25 SUMMARY OF THE INVENTION

The present invention is directed to a novel DNA sequence for an isolated gene (designated ING1). The DNA sequence, an RNA sequence identical to or complementary to the DNA sequence; the protein the DNA sequence encodes, p33^{ING1}, and/or fragments or analogs thereof and antibodies 30 which bind to p33^{ING1} which are useful for diagnosing and/or treating cancer.

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One aspect provides a DNA sequence selected from the group consisting of a DNA isolate substantially identical to the p33^{ING1} DNA sequence shown in Figure 2 and a DNA sequence greater than about 10 base pair (bp) in length capable of hybridizing under stringent conditions to the complement of the 5 p33^{ING1} DNA sequence shown in Figure 2. Recombinant expression vectors comprising such DNA isolates and cells transformed with such recombinant expression vectors are also provided.

Another aspect of the invention provides peptide or protein encoded by the DNA sequence substantially identical to the DNA sequence of Figure 2 or a 10 DNA sequence greater than about 10 base pair (bp) in length capable of hybridizing under stringent conditions to the complement of the p33^{ING1} DNA sequence shown in Figure 2.

Another aspect of the invention provides a method for decreasing proliferation of mammalian cells comprising selecting said mammalian cells 15 whose proliferation is to be decreased and increasing the expression of p33^{ING1} in said mammalian cells. It is contemplated that said mammalian cells may be selected from the group consisting of normal cells and cancerous cells. It is further contemplated that the method of decreasing the expression of p33^{ING1} will comprise introducing into said mammalian cells at least one composition 20 selected from the group consisting of p33^{ING1} and nucleotides which code for p33^{ING1}.

Another aspect of the invention provides a method of diagnosing breast cancer comprising obtaining a biological sample comprising mammary cells suspected of being neoplastic and determining whether or not the biological 25 sample contains p33^{ING1} or the DNA which encodes p33^{ING1}, wherein the presence of p33^{ING1} or its DNA denotes non-cancerous cells.

Another aspect of the invention provides a method of diagnosing breast cancer comprising obtaining a biological sample comprising mammary cells suspected of being neoplastic, contacting said biological sample with at least one 30 antibody to p33^{ING1} under conditions wherein antibody binding to p33^{ING1}

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occurs; and detecting whether or not said antibody binds to said cells, wherein binding to said cells indicates that said cells are non-cancerous.

One aspect of the invention provides nucleic acid isolates greater than 10 nucleotides in length which are substantially identical to the DNA sequence of 5 Figure 3 or its complement. Recombinant expression vectors comprising such sequences and cells transformed with such recombinant expression vectors are also provided.

One other aspect of the invention provides for a nucleic acid sequence which encodes the amino acid sequence of Figures 2 or 3 and recombinant 10 expression vectors comprising such sequences and cells transformed with such recombinant expression vectors.

One other aspect of the invention provides for peptides and proteins having p33^{ING1} biological activity. It is contemplated that such peptides will have an amino acid sequence substantially identical to the amino acid sequence 15 set forth in Figures 2 or 3.

Still a further aspect of the invention provides for antibodies to the p33^{ING1} protein.

A further aspect of the invention provides methods for decreasing proliferation of cancer cells in a patient comprising administering an effective 20 amount of the above-described nucleic acid isolates, nucleic acid sequences, proteins or peptides under conditions wherein p33^{ING1} is expressed in the cancer cells. It is contemplated that the cancer is selected from the group consisting of breast and brain cancers.

Another further aspect of the invention provides methods of increasing 25 cell proliferation of mammalian cells by decreasing expression of p33^{ING1} in the cells. It is contemplated that such methods could include the administration of either a single-stranded oligonucleotide comprising a sequence substantially identical to the complement of the cDNA sequence of Figure 3 or the administration of a single or double-stranded oligonucleotide under conditions 30 that a single-stranded oligonucleotide comprising a sequence substantially identical to the complement of the cDNA sequence of Figure 3 is expressed in

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the cells. It is further contemplated that chemical inhibitors of p33^{ING1} activity could also be administered.

A yet further aspect of the invention provides a method for diagnosing cancer comprising obtaining a biological sample comprising cells suspected of being neoplastic and detecting whether or not the biological sample contains only the native ING1 gene, or expresses native ING1 mRNA or p33^{ING1}, wherein the presence of only the native ING1 gene or expression of native ING1 mRNA or p33^{ING1} denotes non-cancerous cells. Preferably the cancer is breast cancer or brain cancer.

10 A still further aspect of the invention provides a kit for the detection of neoplastic cells in a biological sample comprising cells suspected of being neoplastic comprising a solid support for attaching the mRNA from the cell or tissue to be tested and a labelled polynucleotide of at least 10 nucleotides which polynucleotide is substantially identical to the sequence of Figure 3 or its complement.

15 A still further aspect of the invention provides a kit for the detection of neoplastic cells in a biological sample comprising cells suspected of being neoplastic comprising a solid support for attaching the cells, an anti-p33^{ING1} antibody and a detectable label.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a to 1c illustrate the strategy and biological assays used for cloning ING1.

Figure 2 sets forth the partial cDNA sequence of ING1 (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO: 2) of p33^{ING1}.

25 Figure 3 sets forth the complete cDNA sequence of ING1 (SEQ ID NO: 9) and the predicted amino acid sequence (SEQ ID NO: 10) of p33^{ING1}.

Figures 4a to 4c illustrate the effects of P33^{ING1} overexpression.

Figure 5 illustrates the changes in p33^{ING1} protein levels in breast cancer cell lines. Figure 5a is a Western blot. Figure 5b is a picture of the 30 coomassie-blue stained gel of Figure 5a.

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Figure 6a illustrates Western blotting of neuroblastoma cell lines with anti-p33^{ING1} antibody. Figure 6b illustrates a Southern blot of neuroblastoma cell lines for ING1 DNA. Figure 6c illustrates the RT-PCR reaction on a neuroblastoma cell line compared to a control diploid fibroblast.

5 Figure 7 illustrates the level of ING1 mRNA in control (c) tissue, glioblastoma (GB), astrocytoma (AS) and meningioma (MN) tumors as determined by RT-PCR.

Figures 8a and 8b illustrate the expression of ING1 mRNA and p33^{ING1} in proliferation competent (y) and in senescent human fibroblasts (o).

10 Figure 9 illustrates the level of p33^{ING1} protein through the cell cycle. Panel A has anti-cdk2 antibody as a positive control. Panel B shows the results with anti-p33 antibodies. Panel C shows cell cycle profile at each point as determined by FACS.

Figure 10 illustrates the number of cells per colony of cells blocked for
15 ING1 expression.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein relates to the discovery of a novel tumor suppressor gene, designated ING1, expression of which is found in normal mammary epithelial cells and in normal brain cells but found only at lower
20 levels in several breast cancer cell lines and which is absent in a majority of primary brain tumors including gliomas, meningiomas and astrocytomas.

Using a strategy based upon subtractive hybridization of normal and cancerous mammary epithelial cell mRNAs and the selection of genetic suppressor elements [3], a novel gene was isolated encoding a 33 kDa protein
25 that is a potent inhibitor of cell growth. Acute expression of transfected constructs encoding this gene inhibited cell growth as estimated by decreased S-phase fraction and blocked entry into S-phase following needle microinjection. Chronic expression of antisense constructs resulted in tumor induction *in vivo* and in focus formation *in vitro*, and also conferred the ability to grow in soft agar.
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A. Definitions

As used herein the following terms have the following meanings:

"Antibody" means a molecule that binds to a known antigen. An "anti-p33^{ING1} antibody" means an antibody molecule that binds to one or more epitopes of the p33^{ING1} protein.

5 "Antisense" and "Antisense nucleotides" means DNA or RNA constructs which block the expression of the naturally-occurring gene product. For example, in the present invention, use of a DNA construct that produces ING1 antisense RNA blocks the expression of p33^{ING1} by destroying or inactivating 10 ING1 mRNA.

"Biological sample" means a sample of mammalian cells. These cells may be part of a tissue or organ sample obtained, for example, by biopsy, or they may be individual cells, for example, blood cells or cells grown in tissue culture.

15 "Cancerous cell" means a cell in or from a neoplasm. Preferably the cancerous cells is breast cancer, brain cancer, gastric cancer, haematologic neoplasms and head and neck squamous cell carcinomas.

"Breast cancer" means any of various malignant neoplasms of the breast or mammary tissue.

20 "Brain cancer" means any of various malignant neoplasms of the brain, neuroglial cells or meninges.

"Cell cycle" means the cyclic biochemical and structural events occurring during growth of cells. The cycle is divided into periods called : G₀, Gap₁ (G₁), DNA synthesis (S), GAP₂ (G₂), and mitosis (M).

25 "Cell division" means mitosis, i.e., the usual process of cell reproduction.

"Cell-proliferation-inhibiting-peptide compound" means a peptide or peptide-containing compound which inhibits cell proliferation, either *in vitro* or *in vivo*.

30 "Code" or "encode", when used with reference to a nucleotide's relation to a protein, mean the system whereby particular combinations of adjacent

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nucleotides control the insertion of particular amino acids in equivalent places in a protein molecule.

"Expression" means the production of a protein or nucleotide in the cell.

"Growth" means progression through the cell cycle with the result that
5 two daughter cells are formed from each mother cell. "Actively growing"
means that state wherein cells exhibit growth and cell division.

"Hyperplasticity" means an increase in cell number, excluding tumor formation.

"Label" means to incorporate into a compound a substance that is readily
10 detected. Such substances include radioactive substances and fluorescent dyes,
for example.

"Mammalian cell" means a cell in or from a mammal, either in a tissue or organ or in tissue culture.

"Neoplasia" means the process resulting in the formation and growth of
15 an abnormal tissue that grows by cellular proliferation more rapidly than normal,
and continues to grow after the stimuli that initiated the new growth cease.

"Neoplastic" describes the abnormal tissue that grows by cellular proliferation more rapidly than normal, and continues to grow after the stimuli that initiated the new growth cease.

20 "Normal cell" means a non-cancerous cell.

"Proliferation" means growth and reproduction, i.e., division of cells.

"Native" means the nucleic acid of a non-mutated gene or peptide sequence encoded by such a gene as found in a phenotypically normal cell.

"Substantially identical" means that the polynucleotide or nucleic acid of
25 interest is able to hybridize to the complement of the known sequence under stringent conditions. Such stringent conditions preferably require at least 85% identity, more preferably the conditions require at least 90% identity and most preferably the conditions require at least 95% identity. When used in relation to peptides and proteins, "substantially identical" means that the amino acid sequence of the peptides share at least 85% identity, more preferably at least 90% identity and most preferably at least 95% identity.

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B. Synthesis and Methodology

To identify gene products whose inactivation might contribute to the emergence and growth of cancer cells, a novel positive selection procedure that combines subtractive hybridization with an *in vivo* selection assay was used to 5 identify putative growth-suppressor elements. An overview of the strategy used is shown in Figure 1a.

Following a modified subtractive hybridization protocol [4,5], total cDNA from a normal mammary epithelial cell line [6] was hybridized independently with cDNAs from the breast cancer cell lines MCF-7, BT-483, 10 BT-474, Hs-578T, ZR-75, MD-MB-468, MD-MB-435 and BT-20 which were obtained from the American Type Culture Collection. Subtracted cDNA, theoretically containing sequences more highly expressed in the phenotypically normal epithelial cells, was then used as a probe to screen a normal human fibroblast cDNA library.

15 Following screening, 300 cDNA clones were isolated, and their inserts were digested into fragments of 200-800 base pairs. The fragments were then recloned into the retroviral plasmid vector pLNCX [7]. After passage through the packaging line BOSC 23 [3], retroviruses containing the isolated fragments were used to infect normal mouse mammary epithelial cells (NMuMG). The 20 infected cells were subsequently injected into nude mice.

Within 45 days, several mice developed tumors from which the cloned 25 inserts were recovered by amplification using primers specific for pLNCX in polymerase chain reactions (PCR). Two different sequences were isolated from tumors, one of which was subsequently shown to be expressed in the antisense orientation.

The antisense sequence isolated, when introduced into normal fibroblast cells, consistently showed the biological effects of increased cell proliferation in soft agar and in focus forming assays (Fig. 1b and Table 1). This 182 bp fragment represented nucleotides 781 to 963 of the cDNA shown in Figure 2 30 and nucleotides 942 to 1124 of Figure 3. This cDNA encodes a 33 kDa protein

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called p33^{ING1} for INhibitor of Growth. This was formerly designated p33^{IG1} (see U.S. Serial No. 08/569,721 which is incorporated herein by reference in its entirety).

After plating NMuMG cells infected with either control virus or with
5 virus containing an insert of the antisense orientation of ING1 in soft agar, cells receiving the insert formed, on average, at least 50 times the number of colonies as cells infected with virus alone. Similar results were obtained following transfection of the retroviral construct into NIH3T3 cells, where pLNCX containing the insert of the antisense orientation of ING1 resulted in the
10 formation of 2.3 times the number of generally larger foci than vector alone.

These results corroborated the observations made in the nude mouse assay that the ING1 sequence corresponds to a gene whose product plays a significant role in regulating cell growth.

In order to isolate the gene corresponding to the fragment showing
15 biological effects, normal human fibroblast and HeLa cell libraries were screened with the fragment, resulting in the isolation of 11 positive clones. Two clones contained cDNA whose sequence is shown in Figure 2. The complete cDNA sequence (Figure 3) was obtained using rapid amplification of cDNA ends (RACE) by methods known in the art.

20 Comparison of the sequence of p33^{ING1} shown in Figure 3 to the available protein and nucleotide data bases showed no significant homology to any sequence encoding a known protein and very limited similarity to retinoblastoma binding protein 2 (RbBP2) [9] and to several zinc finger transcription factors. Regions of the p33^{ING1} protein that show homology to
25 retinoblastoma binding protein 2 were identified using the Blast program available from the National Centre for Biological Information (address: www.ncbi.nlm.nih.gov).

30 Use of a polyclonal antibody raised against a glutathione-S-transferase (GST) fusion with p33^{ING1} revealed a protein of 33 kDa by Western blot analysis of human and mouse cell extracts (Figure 1c).

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To determine whether the level of p33^{ING1} was decreased in cells infected with viral constructs containing the antisense orientation, lysates were prepared from control NMuMG cells and from NMuMG cells infected with antisense ING1 that had grown and formed colonies in semi-solid medium.

5 Results of Western blot analysis showed that chronic expression of antisense construct reduced the expression of the endogenous p33^{ING1} protein by approximately 90% in the cells (Figure 1c, lane 6) compared to control parental cells (Figure 1c, lane 5).

The ING1 cDNA contains several AU-rich elements (AREs) in the 3' 10 untranslated region of the clone (Figure 2) which are believed to be involved in the destabilization of specific mRNAs [10].

Since the ING1 gene was originally isolated by subtractive hybridization between normal and transformed epithelial cDNAs, the levels of ING1 mRNA expression in different normal, breast cancer, and brain cancer cell lines were 15 examined. Results from Northern blot analysis show that ING1 is expressed at considerably lower levels (approximately 2-8 fold as estimated by scanning densitometry) in BT-20, ZR-75, MDA-MB-435 and T-47D breast cancer cells compared to MDA-MB-468 and SK-BR-3 breast cancer cells and to normal Hs68 fibroblasts. Results from reverse transcription polymerase chain reaction 20 (RT-PCR) showed that ING1 is not expressed or is expressed at very low levels in glioblastomas, astrocytomas and meningiomas.

Isolation of a DNA fragment that was capable of inducing tumors, foci and growth in soft agar when expressed in the antisense orientation, suggested that the cellular role of p33^{ING1} is to negatively regulate growth. To test this 25 idea, part of the ING1 cDNA was cloned into the mammalian expression vector pBK in the sense orientation (pING1-S). This construct and the plasmid vector, both of which contain neomycin resistance genes and a cytomegalovirus (CMV) promoter, were transfected into human breast cancer (Hs578T) and normal fibroblast (Hs68) cells. Following growth of the cells in antibiotic for 3 weeks, 30 a large number of stable transformants were recovered from cells transfected

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with vector (1+3), whereas very few colonies were visible in plates of cells transfected with the sense orientation of ING1 cDNA (2+4)(Figure 4a).

To corroborate the results of these chronic assays, the effect of microinjecting these constructs, together with non-specific antibodies into 5 fibroblasts was examined. Arrows in panels 1 and 3 of Figure 4b identify cells injected with sense (S) and antisense (α S) constructs, respectively, which were visualized by staining for the presence of coinjected non-specific antibodies using indirect immunofluorescence.

Arrows in panels 2 and 4 of Figure 4b show that cells injected with 10 pING1-S failed to incorporate bromodeoxyuridine (BrdU) (panel 2) over a 36 hour time course after injection. In contrast, those injected with pING1- α S entered S phase (panel 4) as estimated by staining with anti-BrdU antibodies.

Figure 4c shows the combined results of 5 separate experiments, which indicated that injection of the pBK vector or of pING1- α S constructs had no 15 appreciable effect upon the ability of injected cells to incorporate BrdU, whereas injection of pING1-S blocked the ability of cells to enter into and proceed through S phase.

Similar results were obtained in larger populations of cells that were 20 electroporated with vector, sense and antisense construct DNAs together with a construct encoding the CD20 surface marker. Such co-transfections allowed the analysis of DNA content in cells that had taken up DNA by staining for CD20 and subsequent analysis by fluorescence activated cell sorting (FACS). As shown in Table 2, the CD20-expressing population co-transfected with pING1-S had, on average, 63.1% of cells in G0/G1 whereas those co-transfected with 25 vector had 33.6% of cells in G0/G1 when cells were fixed and stained 48 hours after electroporation.

These results, using several independent methods, indicate that the 30 overexpression of p33^{ING1} inhibits cell growth and DNA synthesis in both transient and chronic assays, most likely by arresting cells in the G1 phase of the cell cycle.

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Since the activity of the tumor suppressor genes increases in senescent cells [20], p33^{ING1} activity in low and high passage cells was checked. As shown in Figures 8a and 8b, ING1 expression (and the level of the p33^{ING1} protein) increased several-fold when cells approached the end of their *in vitro* replicative lifespan.

These data demonstrate that p33^{ING1} is a novel inhibitor of cell growth and a candidate tumor suppressor. Additional experiments also indicate that p33^{ING1} is localized in the nucleus of cells, which is consistent with p33^{ING1}'s functioning as a tumor suppressor. Further data showed that p33^{ING1} is localized to the 13q33-34 chromosome region. A number of human cancers have been mapped to this region including primary gastric cancer; haematologic neoplasms; head and neck squamous cell carcinomas. Accordingly, it is contemplated that the nucleic acid sequences of the present invention may be used to detect cancerous or neoplastic cells of these types.

Alternatively, p33^{ING1} might play a role in the regulation of cyclin-dependent kinases (CDKs), as reported recently for the family of CDK inhibitors including p18[11], p21[12,13] and the candidate tumor suppressor p16^{MTS1}[8] to which a portion of the p33^{ING1} sequence shows some homology, and which has been reported to be the MTS1 multiple tumor suppressor locus of human chromosome 9p21 that is inactivated in many types of human tumors [14,15].

It is expected that several p33^{ING1}-related peptides will be useful in the present invention. In particular, p33^{ING1}, its analogs and related proteins and peptides which are effective in suppressing the proliferation of cancerous cells are preferred.

Included within the scope of the p33^{ING1}, as that term is used herein, are p33^{ING1}'s having the amino acid sequence set forth in Figures 2 and 3, glycosylated or deglycosylated derivatives of p33^{ING1}, homologous amino acid sequence variants of the sequence of Figures 2 and 3, and homologous *in vitro*-generated variants and derivatives of p33^{ING1}, which are capable of exhibiting a biological activity in common with the p33^{ING1} of Figure 3.

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p33^{ING1} biological activity is defined as either: (1) immunological cross-reactivity with at least one epitope of native p33^{ING1}, or (2) the possession of at least one cell proliferation, cell regulatory or tumor suppressive function qualitatively in common with native p33^{ING1}s. One example of the qualitative 5 biological activity of p33^{ING1} is its ability to inhibit cell growth as estimated by decreasing the S-phase fraction in a population of cells.

Immunologically cross-reactive, as used herein, means that the candidate polypeptide is capable of competitively inhibiting the qualitative biological activity of the native p33^{ING1} having this activity, with polyclonal antisera 10 raised against the known active analog. Such antisera are prepared in conventional fashion by injecting goats or rabbits, for example, subcutaneously with the known active analog in complete Freund's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freunds.

This invention is concerned with amino acid sequence variants of native 15 p33^{ING1}. Amino acid sequence variants of the p33^{ING1} are prepared with various objectives in mind, including increasing the affinity of the p33^{ING1} for its binding partner, facilitating the stability, purification and preparation of the p33^{ING1}, modifying its biological half-life, improving therapeutic efficacy, and lessening the severity or occurrence of side effects during therapeutic use of the 20 p33^{ING1}.

Amino acid sequence variants of the p33^{ING1} fall into one or more of three classes: insertional, substitutional, or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the p33^{ING1}, by which DNA encoding the variant is obtained, and 25 thereafter expressing the DNA in recombinant cell culture. However, variant p33^{ING1} fragments having up to about 100 to 150 amino acid residues are prepared conveniently by *in vitro* synthesis.

The amino acid sequence variants of the p33^{ING1} are predetermined variants not found in nature or naturally occurring alleles. The p33^{ING1} 30 variants typically exhibit the same qualitative biological activity as naturally occurring p33^{ING1}. However, the p33^{ING1} variants and derivatives that are not

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capable of exhibiting qualitative biological activity similar to native p33^{ING1}, may nonetheless be useful as reagents in diagnostic assays for p33^{ING1} or antibodies to p33^{ING1}. Further, when insolubilized in accordance with known methods, they may be used as agents for purifying anti-p33^{ING1} antibodies from 5 antisera or hybridoma culture supernatants. Further, they may be used as immunogens for raising antibodies to p33^{ING1} or as a component in an immunoassay kit (labeled so as to be a competitive reagent for native p33^{ING1} or unlabeled so as to be used as a standard for the p33^{ING1} assay) so long as at least one p33^{ING1} epitope remains active in these analogs.

10 While the site for introducing an amino acid variation may be predetermined, the mutation, per se, need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random or saturation mutagenesis (where all 20 possible residues are inserted) is conducted at the target codon and the expressed p33^{ING1} variant is screened for the 15 optimal combination of desired activities. Such screening is within the ordinary skill of the art.

Amino acid insertions will usually be on the order of from about one to about ten amino acid residues; substitutions are typically introduced for single residues and deletions will range from about one to about thirty residues.

20 Deletions or insertions preferably are made in adjacent pairs. That is, a deletion of two residues or insertion of two residues. Substitutions, deletions, insertions or any combination thereof may be introduced or combined to arrive at a final construct.

Insertional amino acid sequence variants of the native p33^{ING1} are those 25 in which one or more amino acid residues extraneous to native p33^{ING1} are introduced into a predetermined site in the target p33^{ING1} and which displace the pre-existing residues. Commonly, insertional variants are fusions of heterologous proteins or polypeptides to the amino or carboxyl terminus of the p33^{ING1}. Such variants are referred to as fusions of the p33^{ING1} and a 30 polypeptide containing a sequence which is other than that which is normally

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found in the p33^{ING1} at the inserted position. Several groups of fusions are contemplated for carrying out the invention described herein.

Immunologically active p33^{ING1} derivatives and fusions comprise the p33^{ING1} and a polypeptide containing a non-p33^{ING1} epitope. Such 5 immunologically active derivatives and fusions of p33^{ING1} are within the scope of this invention. The non-p33^{ING1} epitope may be any immunologically competent polypeptide, i.e., any polypeptide which is capable of eliciting an immune response in the animal in which the fusion is to be administered, or which is capable of being bound by an antibody raised against the non-p33^{ING1} 10 polypeptide.

Substitutional variants are those in which at least one residue in the Figure 3 sequence has been removed and a different residue inserted in its place. Novel amino acid sequences as well as isosteric analogs (amino acid or otherwise) are included within the scope of this invention.

15 Some deletions, insertions and substitutions will not produce radical changes in the characteristics in the p33^{ING1} molecule. However, while it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, for example, when modifying an immune epitope on the p33^{ING1} protein, one skilled in the art will appreciate that the effect will be 20 evaluated by routine screening assays. For example, a change in the immunological character of the p33^{ING1} protein, such as affinity for a given antibody, is measured by a competitive-type immunoassay. Modifications of protein properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with 25 carriers or into multimers may be assayed by methods well known to one of skill in the art.

Deletions of cysteine or other labile amino acid residues may also be desirable. For example, they may increase the oxidative stability of the p33^{ING1} protein. Deletion or substitution of potential proteolysis sites, e.g., Arg 30 Arg, is accomplished by deleting one of the basic residues or substituting one with glutaminyl or histidyl residues.

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Covalent modifications of the p33^{ING1} protein are included within the scope of the present invention. Such modifications are introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or terminal amino acid residues. The 5 resulting covalent derivatives of p33^{ING1} are useful to identify residues important for p33^{ING1}'s biological activity, for immunoassays of the p33^{ING1} or for preparation of anti-p33^{ING1} antibodies for immunofinity purification of recombinant p33^{ING1}. Such modification are within the ordinary skill of the art and are performed without undue experimentation.

10 In general, prokaryotes are used for cloning of DNA sequences and in constructing the vectors useful in the present invention. For example, *E. coli* HB101, DH5 α and XL1-blue are particularly useful. These examples are meant to be illustrative and do not limit the present invention. Alternatively, *in vitro* methods of cloning such as the polymerase chain reaction may be used.

15 Expression hosts typically are transformed with DNA encoding the p33^{ING1} protein which has been ligated into an expression vector. Such vectors ordinarily carry a replication site, although this is not necessary where chromosomal integration will occur. Expression vectors may also include marker sequences which are capable of providing phenotypic selection in 20 transformed cells. Expression vectors also optimally will contain sequences which are useful for the control of transcription and translation.

Expression vectors used in eukaryotic host cells will also contain sequences necessary for the termination of transcription which may affect mRNA expression. Expression vectors may contain a selection gene as a 25 selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase, thymidine kinase, neomycin or hygromycin.

Antibodies to the p33^{ING1} may be prepared in conventional fashion [18] by injecting goats or rabbits, for example, subcutaneously with the complete p33^{ING1} protein or a peptide consisting of at least 10 amino acids similar to the 30 p33^{ING1} protein in complete Freund's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freund's adjuvant. The

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anti-p33^{ING1} antibodies may be directed against one or more epitopes on p33^{ING1}. Monoclonal antibodies against p33^{ING1} can be prepared by methods known in the art [18]. The antibodies are preferably labelled with a marker, for example, with a radioactive or fluorescent marker. It is contemplated that the 5 antibodies would be labelled indirectly by binding them to an anti-goat or anti-rabbit antibody covalently bound to a marker compound.

C. Pharmaceutical Compositions

The present invention may be used to block the growth or decrease the proliferation of cancer cells by increasing expression of p33^{ING1}. Blocking the 10 growth of cancer cells is of obvious importance. A method of inhibiting cell division, particularly cell division which would otherwise occur at an abnormally high rate, is also possible. For example, increased cell division is reduced or prevented by introducing into cells a drug or other agent which can increase, directly or indirectly, expression of p33^{ING1}.

15 In one embodiment the p33^{ING1} protein or a peptide having p33^{ING1} biological activity is introduced directly. In a preferred embodiment the peptide possesses at least one cell proliferation, cell regulatory or tumor suppressive function qualitatively in common with native p33^{ING1}.

In another embodiment nucleotides coding for p33^{ING1} are introduced by 20 retroviral or other means. In one embodiment the nucleotide coding for p33^{ING1} comprises a nucleotide sequence which codes for the amino acid sequence of p33^{ING1} as set forth in Figure 3. In another embodiment the nucleotide sequence coding for p33^{ING1} comprises a nucleotide sequence which codes for the amino acid sequence set forth in Figure 2. Preferably the 25 nucleotide sequence is substantially identical to the cDNA sequence of Figure 3, more preferably the sequence is substantially identical to the cDNA sequence of Figure 2 and most preferably the sequence is substantially identical to nucleotides 161 to 1143 of the cDNA sequence of Figure 3.

Cell division is increased by preventing transcription of ING1 DNA 30 and/or translation of RNA. This can be carried out by introducing antisense

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oligonucleotides of the ING1 sequence into cells, in which they hybridize to the p33^{ING1}-encoding mRNA sequences, preventing their further processing. It is contemplated that the antisense oligonucleotide can be introduced into the cells by introducing antisense single-stranded nucleic acid which is substantially identical to the complement of the cDNA sequence in Figures 2 or 3. It is also contemplated that an antisense oligonucleotide can be expressed in the cells by introducing a single- or double-stranded polynucleotide into the cell under conditions wherein a single-stranded nucleic acid sequence which is substantially identical to the complement of the cDNA sequence in Figures 2 or 3 is 5 expressed in the cell, for example, by placing the polynucleotide in the antisense direction under the control of a strong promoter. It is contemplated that the antisense oligonucleotide introduced to the cell or expressed in the cell is at 10 least 100 nucleotides, more preferably it is at least 200 nucleotides and most preferably it is at least 400 nucleotides in length. Most preferably the antisense 15 oligonucleotide sequence is substantially identical to the complement of nucleotides 942 to 1124 of the cDNA sequence set forth in Figure 3.

It is also possible to inhibit expression of p33^{ING1} by the addition of agents which degrade p33^{ING1}. Such agents include a protease or other substance which enhances p33^{ING1} breakdown in cells. In either case the effect 20 is indirect, in that less p33^{ING1} is available than would otherwise be the case.

Viral or plasmid vectors may be used to deliver sense and antisense constructs to target cells *in vivo*. Such viral vectors may include retroviruses, adenovirus or adenovirus-associated viruses. Such methods are known in the art [19].

25 Parenteral administration of the nucleic acids is preferred with subdermal or intramuscular administration most preferred. Intravenous administration or use of implanted milliosmol pumps (available from Alza) may also be used.

When used for parenteral administration, which is preferred, the nucleic acids of the present invention may be formulated in a variety of ways. Aqueous 30 solutions of the nucleic acids of the present invention may be encapsulated in polymeric beads, liposomes, nanoparticles or other injectable depot formulations

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known to those of skill in the art. (Examples thereof may be found, for example, in Remington's Pharmaceutical Sciences, 18th Edition, 1990.) The nucleic acids may also be encapsulated in a viral coat. Doses are selected to provide effective inhibition of cancer cell growth and/or proliferation.

5 The methods of this invention may also be achieved by using a pharmaceutical composition comprising one or more of the following cancer cell proliferation inhibiting compounds: p33^{ING1}, its analogs and related proteins and peptides. Doses are selected to provide effective inhibition of cancer cell growth and/or proliferation.

10 Parenteral administration of the proteins or peptides is preferred, with subdermal or intramuscular administration most preferred. Intravenous administration or use of implanted milliosmol pumps (available from Alza) may also be used.

15 When used for parenteral administration, which is preferred, the proteins and peptides of the present invention may be formulated in a variety of ways. Aqueous solutions of the proteins or peptides of the present invention may be encapsulated in polymeric beads, liposomes, nanoparticles or other injectable depot formulations known to those of skill in the art. (Examples thereof may be found, for example, in Remington's Pharmaceutical Sciences, 18th Edition, 1990.)

20 Compositions including a liquid pharmaceutically inert carrier such as water may also be considered for both parenteral and oral administration. Other pharmaceutically compatible liquids may also be used. The use of such liquids is well known to those of skill in the art. (Examples thereof may be found, for example, in Remington's Pharmaceutical Sciences, 18th Edition, 1990.)

25 The dose level and schedule of administration may vary depending on the particular p33^{ING1}-related compound(s) and/or compositions used, the method of administration, and such factors as the age and condition of the subject.

30 As discussed previously, parenteral administration is preferred, but formulations may also be considered for other means of administration such as

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orally, per rectum, and transdermally. The usefulness of these formulations may depend on the particular compound used and the particular subject receiving the p33^{ING1}-related compound.

Oral formulations of p33^{ING1}-related compounds may optionally and
5 conveniently be used in compositions containing a pharmaceutically inert carrier,
including conventional solid carriers, which are conveniently presented in tablet
or capsule form. Formulations for rectal or transdermal use may contain a
liquid carrier that may be oily, aqueous, emulsified or contain certain solvents
suitable to the mode of administration. Suitable formulations are known to
10 those of skill in the art. (Examples thereof may be found, for example, in
Remington's Pharmaceutical Sciences, 18th Edition, 1990.)

D. Use of ING1 DNA and RNA and p33^{ING1} and Related Proteins and
Peptides for Diagnosis

The present invention also has diagnostic use, since simple
15 immunochemical staining of cells or sections of cells should give an accurate
estimate of the portion of cells expressing p33^{ING1}. Such a test based on the
use of anti-p33^{ING1} antibodies or ING1 polynucleotides and other standard
secondary techniques of visualization will be useful in cancer diagnosis. Such a
test of tumor suppressor gene expression might also be useful to the scientific
20 research community.

Antibodies specifically reactive with p33^{ING1} can be produced, using
known methods [18]. For example, anti-p33^{ING1} antisera can be produced by
injecting an appropriate host (e.g., rabbits, mice, rats, pigs) with p33^{ING1} and
withdrawing blood from the host animal after sufficient time for antibodies to
25 have been formed. Monoclonal antibodies can also be produced using known
techniques [18]. Such antibodies to p33^{ING1} will generally be detectably
labelled (e.g., with a radioactive label, a fluorescent material, biotin or another
member of a binding pair or an enzyme) by methods known in the art. It is
also contemplated that the anti-p33^{ING1} antibodies may be indirectly labelled by

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binding to another second antibody which second antibody is detectably labelled.

In a diagnostic method of the present invention, cells obtained from an individual or a culture are processed in order to determine the extent to which p33^{ING1} is present in cells, in a specific cell type or in a body fluid. This can be determined using known techniques and an antibody specific for p33^{ING1}. Comparison of results obtained from cells or a body fluid being analyzed with results obtained from an appropriate control (e.g., cells of the same type known to have normal p33^{ING1} levels or the same body fluid obtained from an individual known to have normal p33^{ING1} levels) is carried out. Decreased p33^{ING1} levels are indicative of an increased probability of abnormal cell proliferation or oncogenesis or of the actual occurrence of abnormal proliferation or oncogenesis. It is contemplated that the levels of p33^{ING1} in cancerous cells will be at least 50% less than the level of p33^{ING1} in non-cancerous cells, more preferably the levels will be less than 30% of normal levels, most preferably p33^{ING1} will not be expressed.

It is contemplated that a diagnostic kit could include a solid support for attaching the cell or tissue to be tested and a detectably labelled anti-p33^{ING1} antibody. It is further contemplated that the anti-p33^{ING1} antibody may not be labelled but the kit would additionally contain another detectably labelled antibody capable of binding to the anti-p33^{ING1} antibody.

A hybridization probe comprising RNA, ING1 cDNA or ING1 genomic DNA having a sequence substantially identical to Figure 3 ("ING1 polynucleotide") may be employed as a means for determining the sequence of the ING1 gene present in the genomic DNA of a given sample, or the level of ING1 mRNA expressed in cells of such sample. Such hybridization probes will generally be detectably labelled (eg. with a radioactive label, a fluorescent label, biotin, etc). It is also contemplated that the ING1 polynucleotide may be indirectly labelled by methods known in the art.

30 A tissue sample or cell sample can be prepared by conventional means and probed with the labelled ING1 polynucleotide probe to determine the level

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of expression of ING1 mRNA in the cells. The ING1 polynucleotide probe may also be used to determine whether the genomic DNA from the cell sample has a mutation or rearrangement of its ING1 gene by methods known in the art (i.e. PCR sequencing or restriction fragment length polymorphism analysis). The 5 polynucleotide probe may also be used to determine whether the genomic DNA from the cell sample has a mutation/deletion rearrangement of the chromosome region of 13q33-34.

The oligonucleotide probe useful in these methods may comprise at least about 20 nucleotides which sequence is substantially identical to the sequence of 10 Figure 3, more preferably it will comprise at least about 100 nucleotides, and most preferably it will comprise at least 400 nucleotides. In the case of PCR sequencing it is contemplated that one of the two ING1 oligonucleotide primers will be substantially identical to one region of the sequence of Figure 3 and that the second oligonucleotide primer will be substantially identical to the 15 complement of a second region of the sequence of Figure 3. The size of these primers is preferably from 5-25 nucleotides, more preferably from 10-20 nucleotides. Most preferably the oligonucleotide probes and primers will be substantially identical to the coding region of the cDNA sequence of Figure 3. Such nucleotides can be generated synthetically by conventional means.

20 Comparison of the results obtained from cells or a body fluid being analyzed with results obtained from an appropriate control (eg. cells of the same type known to have abnormal or native p33^{ING1} or fluid from an individual known to have normal p33^{ING1}) is carried out. Decreased ING1 mRNA levels are indicative of an increased probability of abnormal cell proliferation or 25 oncogenesis or of the actual occurrence of abnormal proliferation or oncogenesis. It is contemplated that the levels of ING1 mRNA in cancerous cells will be at least 50% less than the level of ING1 mRNA in non-cancerous cells, more preferably the levels will be less than 30% of normal levels, most preferably ING1 mRNA will not be expressed.

30 The presence of a mutation/deletion in one copy of the ING1 gene in a diploid cell is also indicative of an increased probability that abnormal cell

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proliferation or oncogenesis will occur. The presence of mutations/deletions in both copies of the ING1 gene is indicative of possible or actual oncogenesis.

The following examples are offered to illustrate this invention and are not meant to be construed in any way as limiting the scope of this invention.

5 EXAMPLES

The methods described as follows were used to perform the studies described herein. In addition, the generally known methods set forth in laboratory manuals for molecular cloning and antibody techniques [e.g., 17,18] may advantageously be used by one of skill in the art to produce additional 10 embodiments of the invention.

Example 1

Strategy for Cloning and Biological Assays

Subtractive hybridization of breast cancer cell line cDNAs with cDNA from normal mammary epithelial cells, subcloning of subtracted cDNAs into the 15 pLNCX retroviral vector [7] and injection into nude mice was done essentially as described [3] with the modifications noted below. The cloning of full length cDNA was done using standard methods [17]. The strategy is shown in Figure 1a.

cDNA was prepared from an non-transformed mammary epithelial cell 20 line (184A1) [6] and digested with the restriction enzyme Sau3A. cDNAs from the breast cancer cell lines MCF-7, BT-483, BT-474, Hs-578T, ZR-75, MD-MB-468, MD-MB-435 and BT-20 (obtained from the American Type Culture Collection, Bethesda MD) were also digested with Sau3A. Fragments of tester DNA (cDNA from normal epithelial cells) were ligated to "a" adaptors. 25 Fragments of driver DNA (cDNA from breast tumor cells) were ligated to "b" adaptors. Adaptors were prepared by annealing the synthetic oligonucleotides:

5'-GACCTGGCTCTAGAATTCACGACA-3' (SEQ ID NO: 3) with
5'-GATCTGTCGTGAATTCTAGAGCCAGG-3' (SEQ ID NO: 4)
(adaptor "a"); and

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5'-GACTCGACGTTGTAACACGGCAGT-3' (SEQ ID NO: 5) with

5'- GATCACTGCCGTGTTACAACGTCGAG-3' (SEQ ID NO: 6)

(adaptor "b").

The mixture of driver DNA and tester DNA was denatured, then
5 hybridized at 66° C for 18 hours. After hybridization, mixtures were treated
with Mung bean nuclease to eliminate single-stranded adaptor-derived ends from
"heterozygous" hybrids (hybrids containing both a and b adaptors). Resultant
double-stranded molecules were then selectively amplified by PCR using primer
"a".

10 The "amplicons" were then subjected to five successive rounds of
hybridization, selective degradation and PCR amplification using 40 µg of driver
cDNA containing adaptors and 200 ng, 5 ng and 5 pg of tester amplicons in
respective rounds. This procedure allowed for a significant enrichment of
sequences that were more highly expressed in the phenotypically normal
15 epithelial cells as determined by slot blot hybridization.

All subtracted fractions were combined and used as a probe to screen a
near-senescent human diploid fibroblast cDNA library. Following screening,
300 cDNA clones were isolated and their inserts were randomly fragmented
(200-800 bps). These were then ligated with adaptors prepared by annealing
20 two oligonucleotides

5'-AATCATCGATGGATGGATGG-3' (sense) (SEQ ID NO: 22)

5'-CCATCCATCCATCGATGATTAAA-3' (SEQ ID NO: 23) and

were amplified by PCR using the "sense" strand of the adaptor as the PCR

primer. PCR amplified DNA was recloned into the ClaI site of the retroviral

25 plasmid vector pLNCX [7] with synthetic adaptors carrying initiation codons in
all reading frames. This library of about 10⁵ clones, enriched in tumor
suppressor sequences was then used for the isolation of transforming genetic
suppressor elements (GSEs).

After transfection of the recombinant retroviral plasmids into the
30 packaging line BOSC 23 [25], retroviruses containing the isolated cDNA
fragments were used to infect non-tumorigenic immortalized mouse mammary

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epithelial cells (NMuMG) which were subsequently injected subcutaneously into nude mice. Subcloning into the retroviral vector, packaging into the BOSC 23 virus-packaging cell line and assays using nude mice were performed as described [3].

5 After 45 days, two mice developed tumors from which two cDNA inserts were recovered by PCR, one of which is subsequently shown to be expressed in the antisense orientation. The primers used in the PCR amplification were:
5'-CCAAGCTTGTACATCGATGGATG-3' (SEQ ID NO: 7) (sense); and
5'-ATGGCGTTAACTTAAGCTAGCTTGCCAAACCTAC-3' (SEQ ID NO: 8)
10 (antisense). The recovered cDNA insert which was in the antisense orientation was digested with ClaI and HindIII and recloned back into the retroviral vector, pLNCX, in the same position and orientation and then tested individually *in vitro*.

NMuMG cells were infected with retrovirus produced from pLNCX
15 vector containing or not containing the ING1 insert (nucleotides 942 to 1,124 of the ING cDNA set forth in Figure 3). The soft agar culture was comprised of two layers: an underlay (DMEM, 10% FCS, 0.6% agar) and an overlay (DMEM, 10% FCS, 0.3% agar). 5×10^4 cells were plated in soft agar in 10 cm plates and were left at 37°C for 6-7 weeks before being counted. 5×10^5
20 transfected NIH 3T3 cells were plated per 10 cm dish. Transfected NIH 3T3 cells were grown in 5% serum for 4 weeks prior to fixing and visualizing foci. pLNCX-S and pLNCX- α S represent sense and antisense orientations of the ING1 cDNA insert, respectively.

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TABLE 1

Results of the soft agar and focus forming assays.

	Trial Number	Soft agar assay				Focus forming assay			
		1	2	3	4	mean	1	2	mean
5	pLNCX (vector)	0	0	0	0	0	9	13	11
	pLNCX-Ras	224	248	208	(-)	226.7	(-)	(-)	(-)
	pLNCX- α S	42	46	41	82	52.8	18	34	26
10	pLNCX-S	(-)	(-)	0	0	0	(-)	(-)	(-)

(-) = not determined

These results showed that the antisense ING1 cDNA insert caused increased cell proliferation.

Panel 1 of Figure 1b shows NMuMG cells infected with the retroviral vector pLNCX and panel 2 of Figure 1b shows cells infected with the retroviral vector pLNCX containing the antisense ING1 insert. The bar equals 1 mm. Panel 3 of Figure 1b shows NIH 3T3 cells transfected with vector alone and panel 4 of Figure 1b shows cells transfected in parallel with pLNCX containing the antisense ING1 insert.

20 Example 2

cDNA of ING1 and Predicted Amino Acid Sequence of p33^{ING1}

In order to isolate the gene corresponding to the fragment showing biological effects, normal human fibroblast and HeLa cell cDNA libraries were screened with the ING1 cDNA fragment from Example 1, resulting in the isolation of 11 positive clones. Two clones containing the largest cDNA inserts were sequenced on both strands using an Applied Biosystems automated sequencer, yielding the sequence shown in Figure 2.

In order to obtain the 5' end of the ING1 gene, 5' RACE (rapid amplification of cDNA ends) was used. Total cDNAs isolated following reverse transcription had the synthetic adaptor

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5'-GTACATATTGTCGTTAGAACGCGTAATACGCCTCACTATAAGGA-3'

(SEQ ID NO: 11) ligated to them and PCR reactions using nested primers from both the adaptor and the ING1 gene were used to amplify the 5' ends of all RT-generated cDNAs. The primers used in the amplification were:

- 5 5'-CTGGATCTTCTCGTCGCC-3' (SEQ ID NO: 12) and
5'-AGTGCAGCATCGGCCGCTTC-3' (SEQ ID NO: 13) from the ING1 sequence and:

5'-GTACATATTGTCGTTAGAACGCG-3' (SEQ ID NO: 14) and
5'-TAATACGCCTCACTATAAGGA-3' (SEQ ID NO: 15) from the adaptor

- 10 sequence. The largest PCR products were recovered from agarose gels following electrophoresis and were subcloned and sequenced to generate the full-length sequence shown in Figure 3.

The predicted coding region of ING1 begins at nucleotide 16 and ends at nucleotide 898, as shown in Figure 3, predicting a translation product of 33,350 daltons. Comparison of the sequence of p33^{ING1} to the available protein and nucleotide data bases showed no significant homology to any sequence encoding a known protein and very limited similarity to retinoblastoma binding protein 2 (RbBP2) [9] and to several zinc finger transcription factors. Regions of the p33^{ING1} protein that show homology to different members of the p16^{MTS1} family of cyclin-dependent kinase inhibitors and to retinoblastoma binding protein 2 were identified using the Blast program available from the National Centre for Biological Information (address: www.ncbi.nlm.nih.gov).

Example 3

- Expression of a GST-p33^{ING1} fusion protein and creation of anti-p33 polyclonal antibody

In order to generate polyclonal antibodies, a fragment of ING1 containing nucleotides 161-1146 of Figure 3 was subcloned into the EcoRI-Xhol sites of the bacterial expression vector pGEX-4T1 (Pharmacia Biotech, Inc., Quebec, Canada) containing the glutathione-binding portion of glutathione-S-transferase (GST). Plasmids were sequenced to verify that the correct reading frame was obtained and the constructs were electroplated into *E.coli* XL1-Blue.

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Following selection, bacterial cultures were induced to express the fusion protein by the addition of 0.1mM isopropyl thio-galactopyranoside (IPTG) and fusion protein was purified by standard glutathione-Agarose column affinity chromatography. Eluted GST-p33^{ING1} fusion protein was dialyzed and used in 5 immunogen in female New Zealand white rabbits. After four boosters, rabbits were bled and their serum tested for reactivity against the fusion protein. All animals showed reactivity and the bleeds showing the highest titer were chosen for subsequent use in Western blot, immunoprecipitation and immunofluorescence protocols.

10

Example 4

Effect of the antisense ING1 fragment on expression of p33^{ING1} in tissue culture cells

Analysis of p33^{ING1} protein levels in cell samples was performed by Western blotting using anti-p33^{ING1} antibodies raised against the GST-p33^{ING1} 15 fusion protein. Proteins were separated by electrophoresis in 12.5% polyacrylamide/SDS gels, and electrophoretically transferred to membranes for 1 hour. The membranes were blocked in TBS (100 mM Tris, 150 mM NaCl) containing 10% non-fat dried milk and 0.1% Tween-20, for 2 hours. Incubation of the membranes with p33^{ING1} antiserum was performed in TBS containing 20 5% nonfat milk and 0.1% Tween 20 (TBST) for 30 minutes. Horseradish peroxidase-conjugated goat anti-rabbit antibody was then applied to the filters for 1 hour in TBST. Peroxidase activity was detected using a chemiluminescence system (Amersham Canada, Oakville Ontario Canada)

As shown in Figure 1c, NMuMG (lane 1) and ZR-75 (lane 2) cell lines 25 were tested. The Western blot analysis of human and mouse cell lysates revealed a protein of 33 kD. Preincubation of antibodies with GST-p33^{ING1} fusion protein blocked recognition of p33^{ING1} in a parallel blot using lysates from the same cells (lanes 3 and 4).

To determine whether the level of p33^{ING1} was decreased in cells 30 infected with viral constructs containing the antisense orientation, lysates were prepared from control NMuMG cells and from NMuMG cells infected with

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antisense ING1 (pLCNX- α S) that had grown and formed colonies in semi-solid medium. A Western blot of lysates from NMuMG cells infected with pLNCX vector (lane 5) or pLNCX vector containing antisense ING1 insert (lane 6) by the method set out above is shown in Figure 1C. Results of the Western blot analysis showed that chronic expression of antisense construct reduced the expression of the endogenous ING1 gene by approximately 90% compared to control parental cells.

Example 5
Effects of p33^{ING1} Overexpression

10 Isolation of a DNA fragment that was capable of inducing foci and growth in soft agar when expressed in the antisense orientation, suggested that the cellular role of ING1 might be to negatively regulate growth. To test this idea, part of the ING1 cDNA (basepairs 161 to 1143 of Figure 3) was cloned into the mammalian expression vector pBK (Stratagene, Aurora, Ontario Canada) in the sense orientation (pING1-S). This construct and the plasmid vector, both of which contain neomycin resistance genes and a cytomegalovirus (CMV) promoter, were transfected into human breast cancer (Hs578T) and normal fibroblast (Hs68) cells. Following growth for 3 weeks in medium containing G418, plates were fixed and stained with Coomassie Brilliant Blue to identify surviving colonies. A large number of stable transformants were recovered from cells transfected with vector whereas very few colonies were visible in plates of cells transfected with the sense orientation of the cDNA of ING1. Figure 4A shows the results when human Hs578T breast cancer cells (panels 1 and 2) and normal fibroblasts (panels 3 and 4) were transfected with the plasmids pBK (panels 1 and 3) or pING1-S containing the ING1 cDNA in the sense orientation (panels 2 and 4).

In order to corroborate the results of these chronic assays, we next examined the effect of microinjecting these constructs on the ability of normal diploid fibroblasts to initiate DNA synthesis.

30 Hs68 cells were plated on glass coverslips, deprived of serum for 12 hours, microinjected with the indicated mixture of plasmid DNA (0.1 μ g/ml)

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plus nonspecific IgG (2 µg/ml) and were then incubated for 36 hours in complete medium containing BrdU. Fixed cells were identified by staining for injected IgG and for the incorporation of BrdU. Microinjection, fixation and staining were done as described previously [16].

5 Figure 4C shows the combined results of 5 separate experiments. Each group represents 110-200 injected cells. As shown in Figure 4B, normal Hs68 HDFs were injected with solutions containing pING1-S plus non-specific rabbit IgG (panels 1 and 2) or with pING1- α S containing the iING1 cDNA in the antisense orientation plus non-specific rabbit IgG (panels 3 and 4). Injected
10 cells were grown in the presence of BrdU and were fixed and stained for the presence of co-injected IgG (panels 1 and 3) or incorporated BrdU (panels 2 and 4). Arrows identify injected cells. Arrows in panels 2 and 4 show that cells injected with pING1-S failed to incorporate bromodeoxyuridine (BrdU, panel 2) over a 36 hour time course after injection, whereas those injected with pING1-
15 α S entered S phase (panel 4) as estimated by staining with anti-BrdU antibodies.
Figure 4C shows the results of 5 separate experiments which indicate that injection of the pBK vector or of pING1- α S constructs had no appreciable effect upon the ability of cells to proceed through S phase.

Similar results were obtained in larger populations of cells that were
20 electroporated with vector, sense and antisense construct DNAs together with a construct encoding the CD20 surface marker. Such co-transfections allowed the analysis by flow cytometry of DNA content in transfected cells that were positive for CD20 staining. Hs68 cells were co-transfected with pCMV-CD20 together with pBK-p33^{ING1}-S or with pBK vector as a negative control. Cells
25 were fixed and stained for CD20 expression using commercially available antibodies and with propidium iodide 48 hours after electroporation. Cell cycle distribution was determined by flow cytometry using fluorescence-activated cell sorting. The percentage of the CD20+ cells in different phases of the cell cycle is shown for two independent experiments.

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TABLE 2

Overexpression of p33^{ING1} arrested cells in G0/G1

	pBK (vector)			pBK-ING1-S		
	G1/G0	S	G2/M	G1/G0	S	G2/M
5	Trial 1	32.7	38.5	28.8	53.3	19.9
	Trial 2	34.5	35.9	29.6	72.8	19.7
	mean	33.6	37.2	29.2	63.1	19.8
mean						
17.2						

As shown in Table 2, the CD20-expressing population that was cotransfected with pING1-S had, on average, 63.1% of cells in G0/G1 whereas those co-transfected with vector had 33.6% of cells in G0/G1 when cells were fixed and stained 48 hours after electroporation. These results, using several independent methods, indicate that the overexpression of ING1 inhibits cell growth in both transient and chronic assays, most likely by arresting cells in the G1 phase of the cell cycle.

15 **Example 6**

Alterations of ING1 in cancer cell lines

Since ING1 was originally isolated by subtractive hybridization between normal and transformed epithelial cDNAs, the ING1 gene and its expression in breast cancer cell lines was also examined. In Figure 5A, lane 1 is MCFIOA phenotypically normal epithelial cell line from mammary gland; lane 2 is MDA-MB-468; lane 3 is ZR-75; lane 4 is BT-20; lane 5 is SK-BR-3; lane 6 is MCFT; lane 7 is Hs578T and lane 8 is BT-474 (breast cancer cell lines). Figure 5B shows the coomassie-blue stained gel corresponding to Figure 5A. The expression of p33^{ING1} in the cell lines was tested by preparing lysates of cell lines and Western blotting using anti-p33^{ING1} antibodies by the method in Example 4. Although analysis of genomic fragments containing the ING1 gene did not reveal any structural changes in breast cell lines, results from Western blot analyses shown in Figure 5 suggest that the p33^{ING1} protein was expressed at considerably lower levels in some breast cancer cells compared to a

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phenotypically normal epithelial cell line. This observation of reduced expression in the absence of mutation is similar to the expression of BRCA-1 reported to occur in non-hereditary forms of breast cancer [25].

Normal diploid control cell strains and neuroblastoma cell lines were analyzed by Western Blot analysis in a manner similar to that set forth for the breast cancer cell lines. Figure 6a illustrates the Western blotting results of IMR-5 (lane 1) SK-L-C6 (lane 2); SK-N-SH (lane 3) all neuroblastoma cell lines, and W138 (lane 4) a normal diploid lung fibroblast cell line. Normal diploid fibroblast cells expressed low levels of p33^{ING1} while immortalized neuroblastoma cells expressed considerably higher levels and in the case of the SK-N-SH neuroblastoma line a truncated protein was observed.

To investigate the nature of the change(s) responsible for truncating p33^{ING1} in this neuroblastoma cell line, two complementary approaches were taken. Southern blot analysis of DNA, from neuroblastomas and from normal fibroblasts that was digested with different restriction endonucleases and probed with a ING1 nucleic acid probe, clearly indicated that p33^{ING1} was rearranged in the neuroblastoma cell line. Human genomic DNAs were digested with HindIII, DraI or PstI, electrophoresed through a 0.7% agarose gel, transferred to a nitrocellulose membrane and hybridized with [³²P]- labelled p33^{ING1} cDNA. Hybridization was performed using standard procedures [17]. Lanes 1-6 show the results for neuroblastoma SK-N-SH (2, 4 and 6) and for normal diploid W138 cells (1, 3 and 5). Patterns such as those shown by W138 cells were also seen in other normal diploid cell strains.

To confirm that changes in the p33^{ING1} gene had occurred in the neuroblastoma cell line and to determine their nature by an independent method, reverse transcription polymerase chain reaction (RT-PCR) with RNA from SK-N-SH neuroblastoma cell line and from a phenotypically normal epithelial cell line (MCF-10) was performed as described [20]. Neuroblastoma cDNA was amplified with PCR primers specific for the p33 gene (direct(d) and reverse (r) primers). These are numbered and shown underlined in Figure 3 and the PCR products were compared with PCR fragments generated in parallel from control

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cell cDNA. Figure 6c, lanes 1 (primers 1d-4r), 3 (1d-2r), 5 (2d-4r) and 7 (2d-3r) show the results for W138, and lanes 2 (1d-4r), 4 (1d-2r), 6 (2d-4r) and 8 (2d-3r) show the results for the neuroblastoma cell line. Primers were
1d: GTAGCGCAGTCTGACAAGCC (nucleotides 474-494 of SEQ ID NO: 9)
5 2d: TGGTTCCACTTCTCGTGCCT (763-782 of SEQ ID NO: 9)
2r: ACGCACGAGAAGTGGAACCA (SEQ ID NO: 16)
3r: TTTGGATTCTCCAGGGCTT (SEQ ID NO: 17) and
4r: TACCTGTTGTAAGCCCTCTC (SEQ ID NO: 18). M shows a 1 kb ladder molecule weight marker. All primer pairs gave similar results in both cell lines
10 except for those using primers beyond nucleotide 858. For example, using primers 3r and 4r give no PCR product using neuroblastoma cDNA which is consistent with data indicating that a deletion or a rearrangement had occurred within the p33^{ING1} gene. These experiments corroborate the idea that the 3' region of the p33^{ING1} gene was mutated in this neuroblastoma.
15 Normal diploid control cell strains and brain cancer cell lines were analyzed by RT-PCR analysis. Reverse transcription with total RNA from each of the cell lines was performed by the method set out in Example 9. The same primer pairs set forth in Example 9 were used. Figure 7 illustrates the RT-PCR results of glioblastoma (lanes GB1-GB4) astrocytoma (lanes AS1-AS3) and meningioma (MN1-MN3) as compared to a control cell line (C1-C2). The ING1 mRNA was expressed at considerably lower levels, or not expressed at all, in the glioblastomas, astrocytomas and meningiomas as compared to the normal cell line.
20
25

Example 7

Nuclear Localization of p33^{ING1}

The experiments described below were performed with a rabbit polyclonal antibody (α p33) which was raised against a bacterially expressed glutathione-S-transferase (GST)-p33^{ING1} fusion protein and which reacted with a 33 kDa protein in human and mouse cell lysates as prepared by the method in
30 Example 3.

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In the first series of experiments, we determined the location of p33^{ING1} in fibroblasts by examining the staining pattern of anti-p33 antibody in fibroblasts by indirect immunofluorescence. For indirect immunofluorescence normal human diploid fibroblasts (Hs68 cells) were grown on glass coverslips 5 for 48 hours at 37°C to 60% confluence. The cells were fixed in 3.7% formaldehyde, washed in 0.5% Triton X-100 and in 0.05% Tween 20 for 10 minutes each at room temperature. Formaldehyde and detergents were diluted in phosphate buffered saline (PBS) pH 7.5. The cells were incubated with a 1:100 dilution of rabbit p33^{ING1} antiserum for 30 min, washed in PBS with 10 0.05% Tween, incubated with goat anti-rabbit IgG-biotin antibody and then with streptavidin conjugated Texas Red [16]. Samples were examined with a Zeiss Axiophot fluorescence microscope and images were photographed on Kodak TMAX 100 film.

Staining with polyclonal rabbit antibody alone was observed both in 15 nuclear and cytoplasmic compartments. Similar results were obtained with anti-p33 antibodies which were preincubated with 5 µg of GST protein indicating that the signal was specific for p33^{ING1}. When the anti-p33 serum was preincubated with 5 µg of GSTp33 fusion protein, nuclear staining was lost completely but cytoplasmic staining remained, indicating that the vast majority 20 of p33^{ING1} staining was nuclear.

To confirm the nuclear localization of the 33 kDa protein. The pING1-s construct of Example 5 was microinjected into normal Hs68 fibroblast cells which were fixed and stained with anti-p33-antibody 24 hours after injection. Strong staining was clearly localized to the nucleus. These results corroborate 25 staining patterns in uninjected cells and show that p33^{ING1} is localized primarily, and possibly exclusively, throughout the nucleoplasm.

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Example 8

Chromosomal Localization of the *ING1* gene

To identify the chromosomal localization of the *ING1* gene, a genomic 18-kb DNA insert containing the gene was labelled with digoxigenin-dUTP and
5 hybridized to synchronized human lymphocyte metaphase spreads.

A genomic clone of the *ING1* gene was isolated from a lambda FIX II
placental human genomic library (Stratagene, Aurora, Ontario, Canada) with
nucleotides 161 to 1143 of the *ING1* sequence of Figure 3 using high stringency
(65°C 0.1X SSC, 0.1% SDS) screening. The identity of the clone was confirmed
10 by partial sequence analysis.

FISH was performed using established methods on
methotrexate/thymidine synchronized, phytohemagglutinin stimulated, normal
peripheral blood lymphocytes [21]. Approximately 50 metaphase spreads were
examined for probe localization. Suppression for 30 minutes with a mixture of
15 sonicated human DNA (Sigma Diagnostics, Mississauga, Ontario, Canada) and
cotl DNA (Gibco/BRL, Burlington, Ontario, Canada) was required to reduce the
background. The stained slides were counterstained with DAPI and actinomycin
D (for a DA-DAPI banding pattern) and were mounted in antifade medium and
visualized utilizing a Zeiss Axioplan 2 microscope. Images of representative
20 mitoses were captured using a cooled CCD camera (Photometrics PXL 1400).
Digital alignment of the images from each fluor was done after registration
calibration through a triple bandpass filter (FITC/Texas Red/DAPI) to minimize
registration error, utilizing commercial software (Electronic Photography v1.3,
Biological Detection Inc., Pittsburgh PA).

25 The results clearly showed localization of the probe to chromosomal
region 13q33-34. At least one specific probe signal was present in more than
90% of the mitoses examined. Approximately 80% of the cells had two
chromatids of a single chromosome. Approximately, 40% showed labelling of
both chromatids of both chromosomes. More than 90% of the signals were
30 localized to a single band. In addition, cohybridization of p33^{ING1} with a

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commercial 13/21 alpha-satellite probe (Oncor, Gaithersberg MD) showed hybridization to the same chromosome.

The *ING1* gene has been localized to an area near known sites of genomic alteration in several human cancers: primary gastric cancer [22], hematologic neoplasms [23] and head and neck squamous cell carcinomas [24].

Example 9

Expression levels of ING1 in young and senescent fibroblasts.

The normal human diploid fibroblast cell strain Hs68 (ATCC CRL#1635) and a phenotypically normal mouse epithelial cell line from mammary gland (NMuNG) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Hs68 cells were used at 30 ("young"), 70 ("pre-aged") and 80 ("old") mean population doublings (MPDs) for expression and life span experiments. After retroviral infection, the human diploid fibroblast cells (HDFs) were repeatedly passaged in 10 cm plates, splitting at a ratio of 1:2 when confluent.

For infection of fibroblasts, the retroviral vector (pLNCX) was used. The highly efficient ecotropic (BOSC23) and amphotropic (CAK8) packaging cell lines were used [26]. pLNCX- α S or pLNCX alone, were transfected into the BOSC23 virus-packaging cell line. Ecotropic and amphotropic packaging lines, and the retroviral vector were kindly provided by Dr. A. Gudkov (University of Illinois at Chicago). The amphotropic cells were infected by viruses from the BOSC23 supernatant. Fibroblasts were plated at 10^5 cells per 10 cm plate and infected with undiluted viral supernatant from amphotropic producer cells. Infection efficiencies ranged from 85 to 95% in individual trials.

Since the activity or expression levels of several tumor suppressors increase in senescent cells, the levels of ING1 expression in low and high passage cells were checked. All experiments were performed on the Hs68 strain of primary normal human diploid fibroblasts. Senescent cells were obtained by passaging early-passage ("young") fibroblasts continuously to a point at which one population doubling required from 2 - 3 weeks to complete compared to 24 hours, on average, for young HDFs. Hs68s at 80 MPDs exhibited characteristics

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typical of senescent cells, including an inability to respond to growth factors and altered morphology including increased size and decreased saturation density.

To study the level of expression of ING1 mRNA, RT-PCR using total RNA isolated from young and old cells was performed (Figure 8A). The relative levels of ING1 transcript were compared to the internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using PCR primers specific for the p33 and GAPDH genes. ING1 and GAPDH were amplified in the same reaction tube using the "primer dropping" approach [27] which internally controls for efficiency of reverse transcription and amplification by PCR.

Reverse transcription (RT) with 1 µg of total RNA from young and old Hs68 cells was performed using 50 U of RNasin (Pharmacia Biotech, Inc., Quebec Canada) and 200 U of MMLV reverse transcriptase for 50 min. at 42°C in 20 µl reaction volumes. Two µl of each RT reaction was amplified using 2 U of Taq polymerase. The two sets of primer pairs for the ING1 gene and for the GAPDH gene that were used, were:
5'-GAAGCGCGGGATGCTGCACT-3'(SEQ ID NO: 19); and
5'-ACGCACGAGAACGTGGAACCA-3'(SEQ ID NO: 16) for the ING1 gene and
5' CGGAGTCAACGGATTGGTCGTAT -3'(SEQ ID NO: 20); and
20 5' - AGCCTTCTCCATGGTGGTGAAGAC 3'(SEQ ID NO: 21) for the GAPDH gene. Thirty two PCR cycles for ING1 and twenty two PCR cycles for GAPDH were performed using standard conditions [17]. Primers for GAPDH were added to PCR tubes at the end of the 10th cycle [27].

The levels of ING1 mRNA were estimated by scanning densitometry to be approximately ten fold higher in senescent fibroblasts compared to young fibroblasts. In order to see if increased mRNA levels resulted in increased protein levels, Western blotting experiments were performed with a rabbit polyclonal antibody that was raised against a bacterially expressed glutathione-S-transferase (GST)-p33^{ING1} fusion protein and that reacted with a 33 kDa protein in human and mouse cell lysates.

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Hs68 and NMuMG cells were harvested and 20 µg of total protein was used in each lane. Proteins were separated by electrophoresis in 12.5% polyacrylamide/SDS gels, and transferred to membranes for 1 hour using an electroblotter. The membranes were blocked in TBS(100 mM Tris, 150 mM NaCl) containing 10% nonfat dried milk and 0.1% Tween-20 for 2 hours.

5 Incubation of the membranes with p33^{ING1} antiserum was performed in TBS containing 5% nonfat milk and 0.1% Tween-20 for 1 hour and then membranes were washed with TBST solution for 30 minutes. Horseradish peroxidase-conjugated goat anti-rabbit antibody was then applied to the filters

10 for 1 hour in TBST. Peroxidase activity was detected using ECL (Amersham Canada, Oakville, Ontario, Canada) and relative band intensities were determined by scanning densitometry.

15 As shown in Figure 8B, the level of p33^{ING1} protein increases approximately 8 fold when cells approach the end of their *in vitro* replicative lifespan, consistent with results obtained using RT-PCR.

Since ING1 appears to arrest cells in G1 when overexpressed and senescent cells are primarily arrested in the G1 phase of the cell cycle [28], the level of p33^{ING1} protein was tested during the cell cycle. Quiescent, proliferation-competent NMuMG cells were serum stimulated, lysates were prepared at different times after serum addition, and samples were analyzed by Western blotting with anti-p33 antibodies by the method set forth above. The level of p33^{ING1} was found to decrease as cells exited from G0, to increase during late G1 and to reach a maximum in S phase. This was followed by a decrease in G2 of the cell cycle (Figure 9B). CDK2 expression was used as a control for cell cycle progression and changed as reported previously (Figure 9A) [29]. Figure 9c shows the results of DNA content analysis by fluorescence-activated cell sorting (FACS) in parallel cultures indicating that cells enter S phase at 16 hours under these experimental conditions. These results indicate that ING1 is regulated following mitogen addition to quiescent cells, with expression reaching a peak during DNA synthesis.

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To determine the effects of reducing the levels of ING1 mRNA on the replicative lifespan of HDFs, cells were infected with a PLNCX- α S (the construct carrying a 182 bp fragment in the antisense orientation and representing nucleotides 942 to 1,124 of the ING1 cDNA (Figure 3)). This 5 antisense fragment effectively inhibits translation of ING1 mRNA as shown previously where chronic expression of the antisense construct resulted in 90% inhibition of the expression of the endogenous p33^{ING1} protein in cells.

Amphotropic and ecotropic packaging cells that were used for infection are capable of producing retroviruses with titers higher than 10⁶ per ml upon 10 transient transfection which allows delivery of the retroviral construct to HDFs with efficiencies of approximately 90% as monitored by expression from a retroviral - β -galactosidase construct.

"Young" HDFs at 30 MPDs were "pre-aged" by continuous subculturing until reaching 70 MPDs. Hs68 cells at 70 mean population doublings (MPDs) 15 were infected with the retroviral vector pLNCX as a control or with pLNCX- α S and were subcultured in parallel using subculturing ratios of 1:2. Infected cells were propagated an additional 10 MPDs after which 10⁵ control PLCNX and 10⁵ PLCNX- α S cells at 80 MPD were split into twelve 10 cm plates and cultivated for two months, with weekly refeeding using complete medium. Some 20 of the cells infected with retrovirus alone were observed to divide once during this time, while cells containing the ING1- α S fragment continued to grow and created visible colonies.

To confirm the effect of the antisense fragment of ING1 in cells, indirect 25 immunofluorescence with a rabbit polyclonal antibody that was raised against p33^{ING1} was performed. Senescent vector-infected fibroblasts and fibroblasts from colonies resulting from ING1- α S retrovirus infection were grown on glass coverslips for 48 hours at 37°C to 60% confluence. Then the cells were fixed in 3.7% formaldehyde, washed in 0.5% Triton X100 and in 0.05% Tween 20 in PBS solution for 10 minutes each at room temperature. The cells were incubated 30 with a 1:100 dilution of rabbit p33^{ING1} antiserum for 30 min, washed in PBS with 0.05% Tween, incubated with goat anti-rabbit IgG-biotin antibody and then

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with streptavidin conjugated Texas Red. Samples were examined with a Zeiss Axiophot fluorescence microscope and images were photographed on Kodak TMAX 400 film.

Staining with anti-p33 antibody was observed in the nuclear compartment
5 of senescent cells containing control virus but not in cells obtained from colonies that had received antisense p33 retrovirus. These results corroborate the previous observations that p33^{ING1} is a nuclear protein and confirmed that the levels of p33^{ING1} protein decrease in cells from colonies resulting from ING1- α S retrovirus infection. Similar results were seen in cells from 3
10 individual colonies and from 20 independent senescent cells containing control retrovirus.

To estimate the efficiency with which down regulation of the ING1 gene by infection with PLCNX- α S was able to extend the proliferative lifespan of normal fibroblasts, the number of cells in each colony was counted. Results of
15 these calculations are shown in Figure 10 in which colonies were divided into 4 groups depending upon the number of cells in the colony. Most colonies contained 100-159 cells, therefore if cells divided in an arithmetic progression (2,4,8...n) this class corresponds to approximately 7 additional MPDs ($2^7=128$). Colonies in the largest category (220-280) correspond to 8 cell doublings
20 ($2^8=256$). Similar results were obtained in two separate trials and strongly indicate that down regulation of p33^{ING1} protein is sufficient to extend the proliferative lifespan of normal fibroblasts by approximately 10%, as previously reported for the p53 tumor suppressor gene [30].

Modification of the above-described modes of carrying out various
25 embodiments of this invention will be apparent to those skilled in the art following the teachings of this invention as set forth herein. The examples described above are not limiting, but are merely exemplary of this invention, the scope of which is defined by the following claims.

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WHAT IS CLAIMED IS:

1. A DNA sequence selected from the group consisting of a DNA isolate substantially identical to the p33^{ING1} DNA sequence shown in Figure 2 and a DNA sequence greater than about 10 base pair (bp) in length capable of hybridizing under stringent conditions to the complement of the p33^{ING1} DNA sequence shown in Figure 2.
2. A recombinant expression vector comprising a DNA isolate according to Claim 1.
3. A cell transformed with a recombinant expression vector according to 10 Claim 2.
4. A peptide or protein encoded by the DNA sequence of Claim 1.
5. A method for decreasing proliferation of mammalian cells comprising:
 - a) selecting said mammalian cells whose proliferation is to be decreased; and
 - b) increasing the expression of p33^{ING1} in said mammalian cells.
6. The method of Claim 5 wherein said mammalian cells are selected from the group consisting of normal cells and cancerous cells.
7. The method of Claim 5 wherein said decreasing said expression of p33^{ING1} comprises introducing into said mammalian cells at least one composition selected from the group consisting of p33^{ING1} and nucleotides which code for p33^{ING1}.

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8. The method of Claim 5 wherein said decreasing proliferation is blocking the proliferation of cancer cells.

9. A method of diagnosing breast cancer comprising:

- 5 a) obtaining a biological sample comprising mammary cells suspected of being neoplastic; and
- b) determining whether or not the biological sample contains p33^{ING1} or the DNA which encodes p33^{ING1}, wherein the presence of p33^{ING1} or its DNA denotes non-cancerous cells.

10. A method of diagnosing breast cancer comprising:

- 10 a) obtaining a biological sample comprising mammary cells suspected of being neoplastic;
- b) contacting said biological sample with at least one antibody to p33^{ING1} under conditions wherein antibody binding to p33^{ING1} occurs; and
- 15 c) detecting whether or not said antibody binds to said cells, wherein binding to said cells indicates that said cells are non-cancerous.

11. A nucleic acid sequence comprising at least 10 nucleotides in length which is substantially identical to the cDNA sequence of Figure 3 or its complement.

20 12. A recombinant expression vector comprising the nucleic acid sequence of Claim 11.

13. A cell transformed with the recombinant expression vector of Claim 12.

25 14. A nucleic acid sequence which encodes the amino acid sequence of Figure 3.

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15. A recombinant expression vector comprising the nucleic acid sequence of Claim 14.

16. A cell transformed with the recombinant expression vector of Claim 15.

5 17. A peptide having p33^{ING1} biological activity.

18. The peptide of Claim 17 having an amino acid sequence substantially identical to the amino acid sequence of Figure 2.

19. The peptide of Claim 17 having an amino acid sequence substantially identical to the amino acid sequence of Figure 3.

10 20. An antibody capable of binding to an epitope of the p33^{ING1} protein.

15 21. A method for decreasing the proliferation of cancer cells in a patient comprising administering an effective amount of the nucleic acid of Claim 14 to the patient under conditions wherein p33^{ING1} is expressed in the patient's cancer cells.

22. The method of Claim 21, wherein the cancer is selected from the group consisting of breast and brain cancer.

20 23. A method for decreasing the proliferation of cancer cells in a patient comprising administering an effective amount of the peptide of Claim 17 to the patient under conditions wherein the peptide is expressed in the patient's cancer cells.

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24. The method of Claim 23, wherein the cancer is selected from the group consisting of breast and brain cancer.

25. A method of increasing cell proliferation by decreasing expression of p33^{ING1} in the cells.

26. The method of Claim 25, further comprising the step of administering to the cell a single-stranded oligonucleotide of at least 100 nucleotides which comprises a sequence substantially identical to complement of the cDNA sequence of Figure 3.

27. The method of Claim 25, further comprising the step of administering to the cell a double-stranded oligonucleotide of which comprises a sequence substantially identical to the cDNA sequence of Figure 3 under conditions such that a mRNA of at least 100 nucleotides having a sequence which is substantially identical to the complement of the cDNA sequence of Figure 3 is expressed.

28. A method for diagnosing cancer comprising:
(a) obtaining a biological sample comprising cells suspected of being neoplastic; and
(b) detecting whether or not the biological sample contains only native ING1 genes
wherein the presence of only native ING1 genes denotes non-cancerous cells.

29. The method of Claim 28, wherein step b further comprises the steps of
(a) isolating the genomic DNA from the cells; and
(b) hybridizing a labelled oligonucleotide of at least 20 nucleotides to the genomic DNA wherein the oligonucleotide is substantially identical to the

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sequence in Figure 3 or its complement under conditions wherein the presence of non-native ING1 genes can be determined.

30. A method for diagnosing cancer comprising:

- (a) obtaining a biological sample comprising cells suspected of being neoplastic; and
- (b) detecting the level of native p33^{ING1} in the biological sample and comparing it to the level of native p33^{ING1} in a non-cancerous cell wherein a decreased level of native P33^{ING1} denotes cancerous cells.

31. A method for diagnosing cancer comprising:

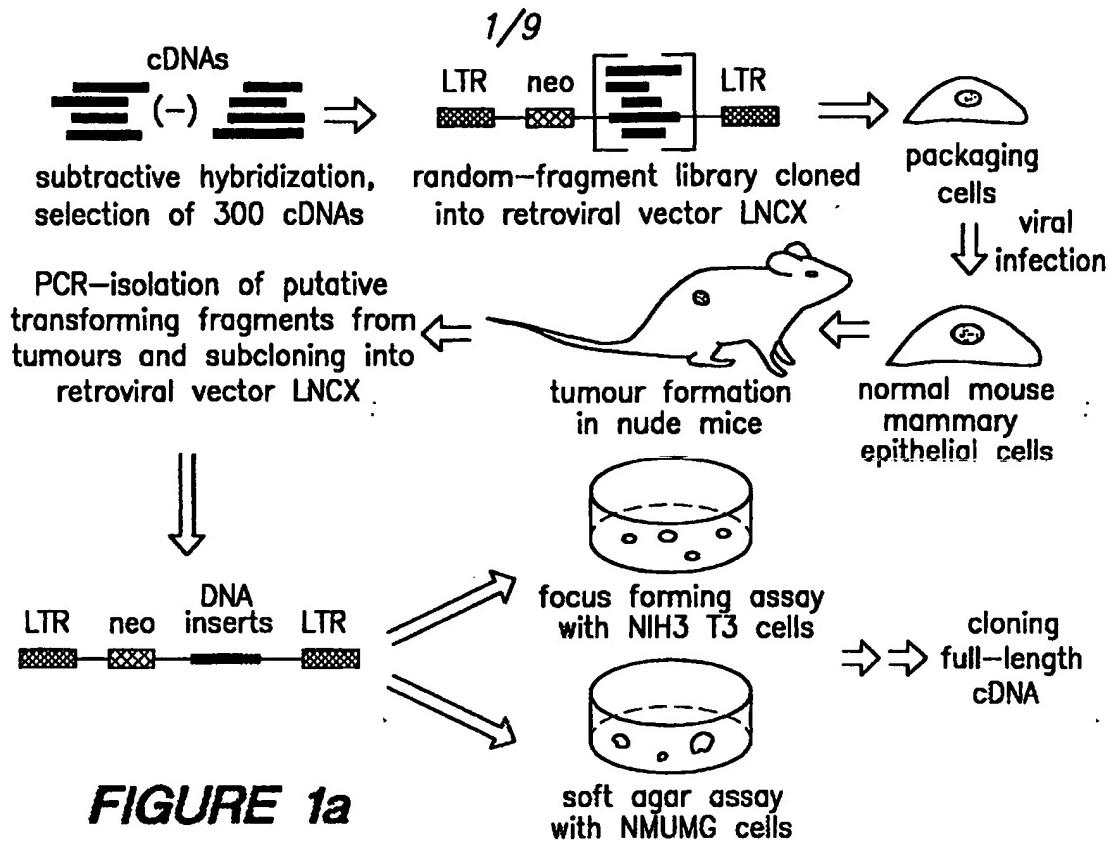
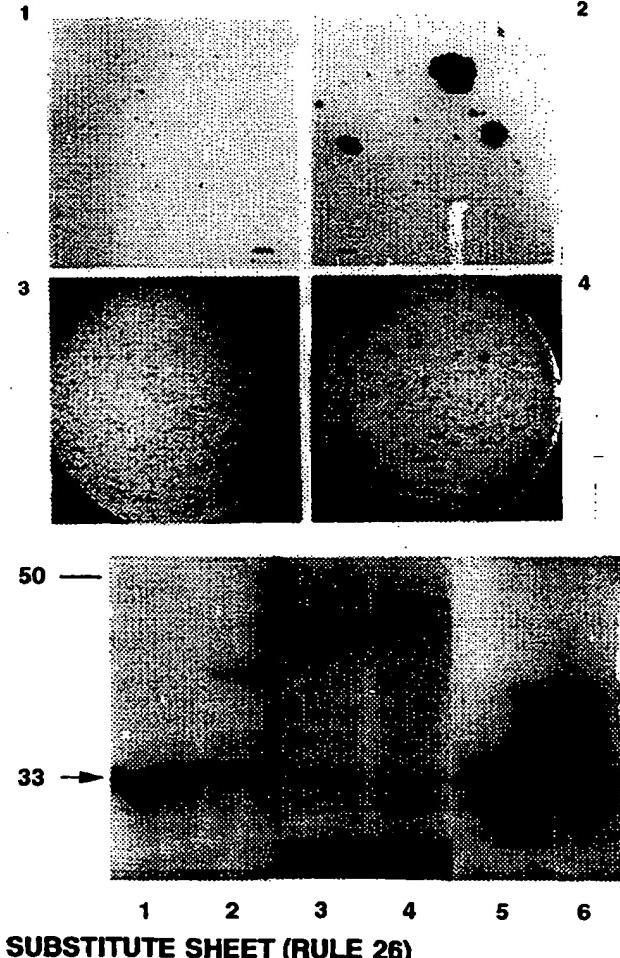
- (a) obtaining a biological sample comprising cells suspected of being neoplastic; and
- (b) detecting the level of ING1 mRNA in the biological sample and comparing it to the level of ING1 mRNA in a non-cancerous cell wherein a decreased level of ING1 mRNA denotes cancerous cells.

32. A kit for the detection of cancer in a biological sample comprising cells suspected of being neoplastic comprising

- (a) a solid support for attaching the mRNA from the cell or tissue to be tested; and
- (b) a detectably labelled polynucleotide of at least 10 nucleotides which polynucleotide is substantially identical to the sequence of Figure 3 or its complement.

33. A kit for the detection of cancer in a biological sample comprising cells suspected of being neoplastic comprising

- (a) a solid support for attaching the cells from the cell or tissue to be tested;
- (b) an anti-p33^{ING1} antibody; and
- (c) a detectable label.

**FIGURE 1a****FIGURE 1b****FIGURE 1c**

SUBSTITUTE SHEET (RULE 26)

2/9

1 CTG ACC CGA GGG TGG GGC CGC GCG TGG CCG TGG AAA CAG ATC CTG AAG GAG CTA GAC GAG
 6! TGC TAC GAG CGC TTC AGT CGC GAG ACA GAC GGG GCG CAG AAG CGG CGG ATG CTG CAC TGT
 1 met leu his cys
 12! GTG CAG CGC GCG CTG ATC CGC AGC CAG GAG CTG GGC GAC GAG AAG ATC CAG ATC GTG AGC
 5 val gln arg ala leu ile arg ser gln glu leu gly asp glu lys ile gln ile val ser
 18! CAG ATG GTG GAG CTG GTG GAG AAC CGC ACG CGG CAG GTG GAC AGC CAC GTG GAG CTG TTC
 25 gln met val glu leu val glu asn arg thr arg gln val asp ser his val glu leu phe
 24! GAG GCG CAG CAG GAG CTG GGC GAC ACA GTG GGC AAC AGC GGC AAG GTT GGC GCG GAC AGG
 45 glu ala gln gln glu leu gly asp thr val gly asn ser gly lys val :gly ala asp arg
 30! CCC AAT GGC GAT GCG GTA GCG CAG TCT GAC AAG CCC AAC AGC AAG CGC TCA CGG CGG CAG
 65 pro asn gly asp ala val ala gln ser asp lys pro asn ser lys arg ser arg arg gln
 36! CGC AAC AAC GAG AAC CGT GAG AAC GCG TCC AGC AAC CAC GAC CAC GAC GGC GGC GCC TCG
 85 arg asn asn glu asn arg glu asn ala ser ser asn his asp his asp asp gly ala ser
 42! GGC ACA CCC AAG GAG AAG AAG GCC AAG ACC TCC AAG AAG AAG AAG CGC TCC AAG GCC AAG
 105 gly thr pro lys glu lys lys ala lys thr ser lys lys lys arg ser lys ala lys
 48! GCG GAG CGA GAG GCG TCC CCT GCC GAC CTC CCC ATC GAC CCC AAC GAA CCC ACG TAC TGT
 125 ala glu arg glu ala ser pro ala asp leu pro lle asp pro asp glu pro thr tyr cys
 54! CTG TGC AAC CAG GTC TCC TAT GGG GAG ATG ATC GGC TGC GAC AAC GAC GAG TGC CCC ATC
 145 leu cys asn gln val ser tyr gly glu met ile gly cys asp asn asp glu cys pro ile
 60! GAG TGG TTC CAC TTC TCG TGC GTG GGG CTC AAT CAT AAA CCC AAG GGC AAG TGG TAC TGT
 165 glu trp phe his phe ser cys val gly leu asn his lys pro lys gly lys trp tyr cys
 66! CCC AAG TGC CGG GGG GAG AAC GAG AAG ACC ATG GAC AAA GCC CTG GAG AAA TCC AAA AAA
 185 pro lys cys arg gly glu asn glu lys thr met asp lys ala leu glu lys ser lys lys
 72! GAG AGG GCT TAC AAC AGG TAG TTT GTG GAC AGG CGC CTG GTG TGA GGA GGA CAA AAT AAA
 205 glu arg ala tyr asn arg ***
 78! CCG TGT ATT TAT TAC ATT GCT GCC TTT GTT GAG GTG CAA GGA GTG TAA AAT GTA TAT TTT
 84! TAA AGA ATG TTA GAA AAG GAA CCA TTC CTT TCA TAG GGA TGG CAG TGA TTC TGT TTG CCT
 90! TTT GTT TTC ATT GGT ACA CGT GTA ACA AGA AAG TGG TCT GTG GAT CAG CAT TTT AGA AAC
 96! TAC AAA TAT AGG TTT GAT TCA ACA CTT AAG TCT CAG ACT GAT TTC TTG CGG GAG GAG GGG
 102! GAC TAA ACT CAC CCT AAC ACA TTA ATT GTG GAA GGA AAA TAT TTC ATT AGC TTT TTT ATT
 108! TTA ATA CAA GTA ATA TTA TTA CTT ATT GAA CAA TTT TTT TTA ATT GGC CAT GTC GCC AAA
 114! AAT ACA GCC TAT AGT AAA TGT GTT TCT TGC TGC CAT GAT GTA TAT CCA TAT AAC AAT TCA
 120! GTA ACA AAG GTT TAA AGT TTG AAG ATT ATT TTT TAA AAA GGT AAA AGG TTA ATT TTT ACA
 126! TGA CAG ATA TTT TAT CTA TTG GCC TGT TCC CCA ATT GGC CAT TTT AAA ATT CTT GGG TAC
 132! ACT TCT CTT AAG TGG TCT AGT CAA GGA ACC TCA AGT CAT GCT TTT GCT ATC ACC AAT CAT
 138! AGT GTA CCC ATC TTT AAT TTA ATT CAG GTG ATT AAA TGT ACA TTT CCA ATT GAA CTT GCA
 144! CTG TAA TAT TAT AAT TGG AAG TGC AGT CAG CAG TAG CTG TCG GAG CTA ATG TCA CAA TTA
 150! TGT GCA AAG GTG TGC TTC CTG CTG TAT GTG AGC TGT AAA ATT GTT ACG TGA AGA ATT AAA
 156! TGA AAC TTG GCC AGT TTG TTC CTC TAG TAG ATT TAA ATT TGA CAT AAG TAA CTT TTA
 162! AAA TTT GTC TTA AAA ATT TAT ACA CCA GCA ATT TAG ACA AAG CCT TAA GCA ATT TTT GTA
 168! TTA TTG TTC TCA CTT ATT ATT AAT AAT GAA GTA GAA GTT ACT TAA TTG CCA GCA ATT AAA
 174! TAC GTG TCA AAA AAG ATT CTG TAT TCA GAC CCC TGG GGT CAG GAA ATT ACT GCC CCA CTT
 180! GTC AAG TTC AGC CCA CCA TCT GTT TGA ACA TTA ATT GAA GTT TAA ATT CTA GTG TCC ATA
 186! AAT AAA GTT TCA GCG GCA CCC CAA AAA AAA AAA AAA AAA AAA

FIGURE 2

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ISA/EP

FIGURE 3

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GAG TAA CCC GAT AAT

16 ATG CCG TTG TGC ACG GCG ACG AGA ATT CCC AGA TAT AGC AGT AGC AGT GAT CCC GGG CCT
 1 met pro leu cys thr ala thr arg ile pro arg tyr ser ser ser ser asp pro gly pro

76 GTG GCT CGG GGC CGG GGC TGC AGT TCG GAC CGC CTC CCG CGA CCC GCG GGG CCG GCT CGG
 21 val ala arg gly arg gly cys ser ser asp arg leu pro arg pro ala gly pro ala arg

136 AGA CAG TTT CAG GCC GCA TCT TTG CTG ACC CGA GGG TGG GGC CGC GCG TGG CCG TGG AAA
 41 arg gln phe gln ala ala ser leu leu thr arg gly trp gly arg ala trp pro trp lys

196 CAG ATC CTG AAG GAG CTA GAC GAG TGC TAC GAG CGC TTC AGT CGC GAG ACA GAC GGG GCG
 61 gln ile leu lys glu leu asp glu cys tyr glu arg phe ser arg glu thr asp gly ala

256 CAG AAG CGG CGG ATG CTG CAC TGT GTG CAG CGC GCG CTG ATC CGC AGC CAG GAG CTG GGC
 81 gln lys arg arg met leu his cys val gln arg ala leu ile arg ser gln glu leu gly

316 GAC GAG AAG ATC CAG ATC GTG AGC CAG ATG GTG GAG CTG GTG GAG AAC CGC ACG CGG CAG
 101 asp glu lys ile gln ile val ser gln met val glu leu val glu asn arg thr arg gln

376 GTG GAC AGC CAC GTG GAG CTG TTC GAG GCG CAG CAG GAG CTG GGC GAC ACA GTG GGC AAC
 121 val asp ser his val glu leu phe glu ala gln gln glu leu gly asp thr val gly asn

436 AGC GGC AAG GTT GGC GCG GAC AGG CCC AAT GGC GAT GCG GTA GCG CAG TCT GAC AAG CCC
 141 ser gly lys val gly ala asp arg pro asn gly asp ala val ala gln ser asp lys pro

496 AAC AGC AAG CGC TCA CGG CGG CAG CGC AAC AAC GAG AAC CGT GAG AAC GCG TCC AGC AAC
 161 asn ser lys arg arg gln arg asn asn glu asn arg glu asn ala ser ser asn

556 CAC GAC CAC GAC GAC GGC GCC TCG GGC ACA CCC AAG GAG AAG AAG GCC AAG ACC TCC AAG
 181 his asp his asp asp gly ala ser gly thr pro lys glu lys lys ala lys thr ser lys

616 AAG AAG AAG CGC TCC AAG GCC AAG GCG GAG CGA GAG GCG TCC CCT GCC GAC CTC CCC ATC
 201 lys lys lys arg ser lys ala lys ala glu arg glu ala ser pro ala asp leu pro lle

676 GAC CCC AAC GAA CCC ACG TAC TGT CTG TGC AAC CAG GTC TCC TAT GGG GAG ATG ATC GGC
 221 asp pro asn glu pro thr tyr cys leu cys asn gln val ser tyr gly glu met ile gly

736 TGC GAC AAC GAC GAG TGC CCC ATC GAG TGG TTC CAC TTC TCG TGC GTG GGG CTC AAT CAT
 241 cys asp asn asp glu cys pro ile glu trp phe his phe ser cys val gly leu asn his

796 AAA CCC AAG GGC AAG TGG TAC TGT CCC AAG TGC CGG GGG GAG AAC GAG AAG ACC ATG GAC
 261 lys pro lys gly lys trp tyr cys pro lys cys arg gly glu asn glu lys thr met asp

856 AAA GCC CTG GAG AAA TCC AAA AAA GAG AGG GCT TAC AAC AGG TAG TTT GTG GAC AGG CGC
 281 lys ala leu glu lys ser lys glu arg ala tyr asn arg ***

916 CTG GTG TGA GGA GGA CAA AAT AAA CCG TGT ATT TAT TAC ATT GCT GCC TTT GTT GAG GTG
 976 CAA GGA GTG TAA AAT GTC TAT TTT TAA AGA ATG TTA GAA AAG GAA CCA TTC CTT TCA TAG

1036 GGA TGG CAG TGA TTC TGT TTG CCT TTT GTT TTC ATT GGT ACA CGT GTC ACA AGA AAG TGG

1096 TCT GTG GAT CAG CAT TTT AGA AAC TAC AAA TAT AGG TTT GAT TCA ACA CTT AAG TCT CAG

1156 ACT GAT TTC TTG CGG GAG GGG GAC TAA ACT CAC CCT AAC ACA TTA AAT GTG GAA GGA

1216 AAA TAT TTC ATT AGC TTT ATT TTA ATA CAA GTC ATA TTA TTA CTT TAT GAA CAA TTT

1276 TTT TTA ATT GGC CAT GTC GCC AAA AAT ACA GTC TAT AGT AAA TGT GTT TCT TGC TGC CAT

1336 GAT GTC TAT CCA TAT AAC AAT TCA GTC ACA AAG GTT TAA AGT TTG AAG ATT ATT TTT TAA

1396 AAA GGT AAA AGG TTA AAT TTT ACA TGA CAG ATA TTT TAT CTA TTG GCC TGT TCC CCA AAT

1456 GGC CAT TTT AAA ATG CTT GGG TAC ACT TCT CTT AAG TGG TCT AGT CAA GGA ACC TCA AGT

1516 CAT GCT TTT GCT ATC ACC AAT CAT AGT GTC CCC ATC TTT AAT TTA TAT CAG GTG TAT AAA

1576 TGT ACA TTT CCA AAT GAA CTT GCA CTG TAA TAT TAT AAT TGG AAG TGC AGT CAG CAG TAG

1636 CTG TCG GAG CTA ATG TCA CAA TTA TGT GCA AAG GTG TGC TTC CTG CTG TAT GTG AGC TGT

1696 AAA AAT GTT ACG TGA AGA AAT AAA TGA AAC TTG GCC AGT TTG TTC CTC TAG TAG TAT ATT

1756 TAA TTT TGA CAT AAG TAA CTT TTA AAA TTT GTC TTA AAA ATT TAT ACA CCA GCA ATT TAG

1816 ACA AAG CCT TAA GCA AAT TTT GTC TTA TTG TTC TCA CTT ATT ATT AAT AAT GAA GTC GAA

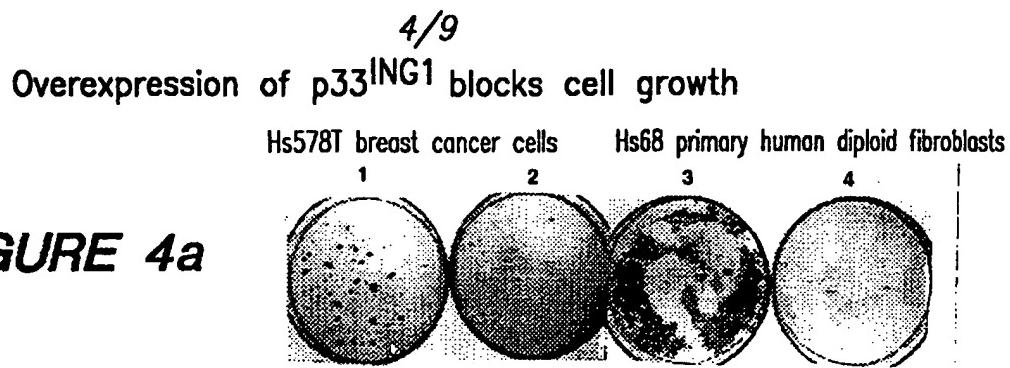
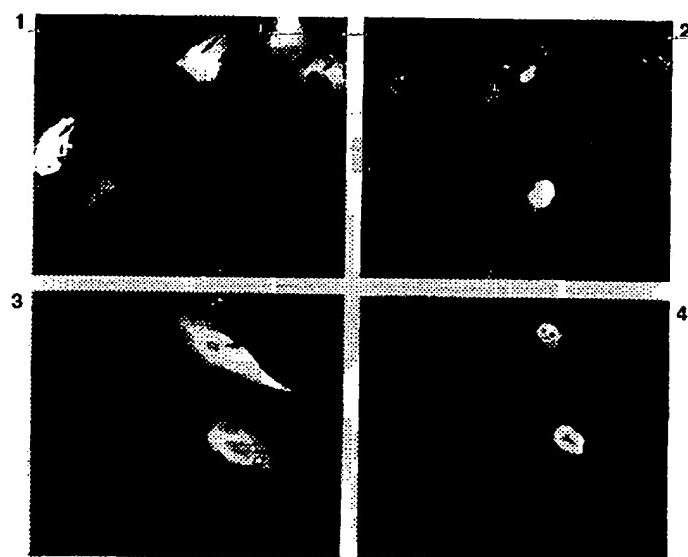
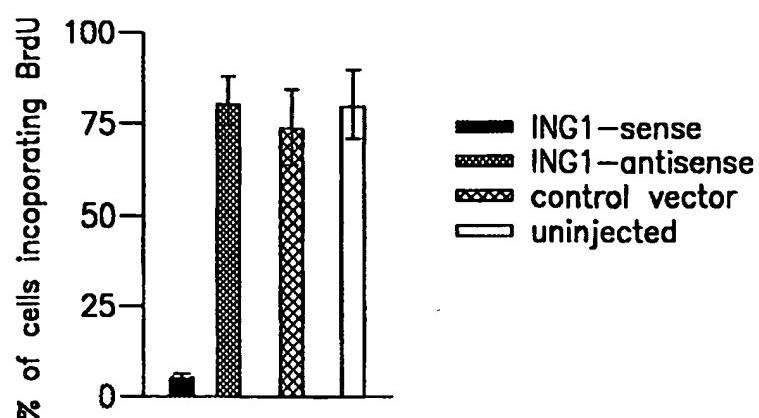
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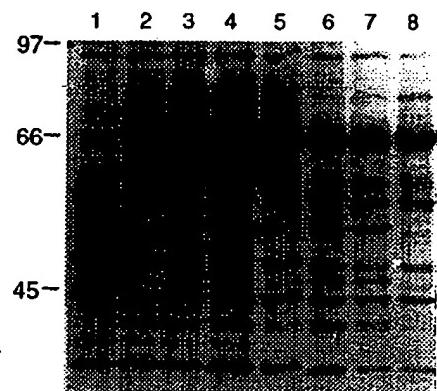
1996 GAA GTT TAA ATT CTA GTG TCC ATA AAT AAA GTT TCA GCG GCA CCC CAA AAA AAA AAA AAA

2056 AAA AAA

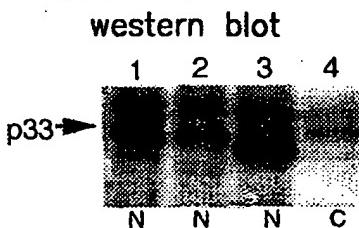
SUBSTITUTE SHEET (RULE 26)

**FIGURE 4a****FIGURE 4b****FIGURE 4c**
SUBSTITUTE SHEET (RULE 26)

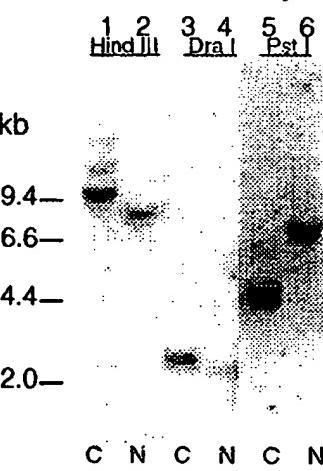
5/9



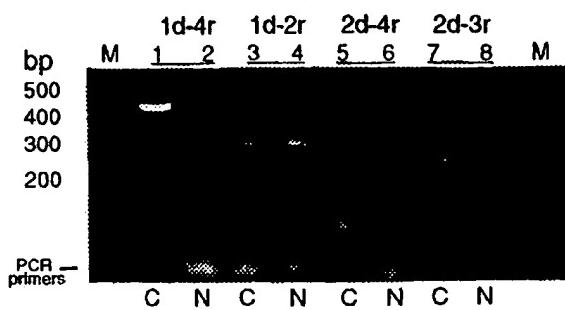
Mutation of the *ING1* gene in neuroblastoma cell lines

FIGURE 6a**FIGURE 6b**

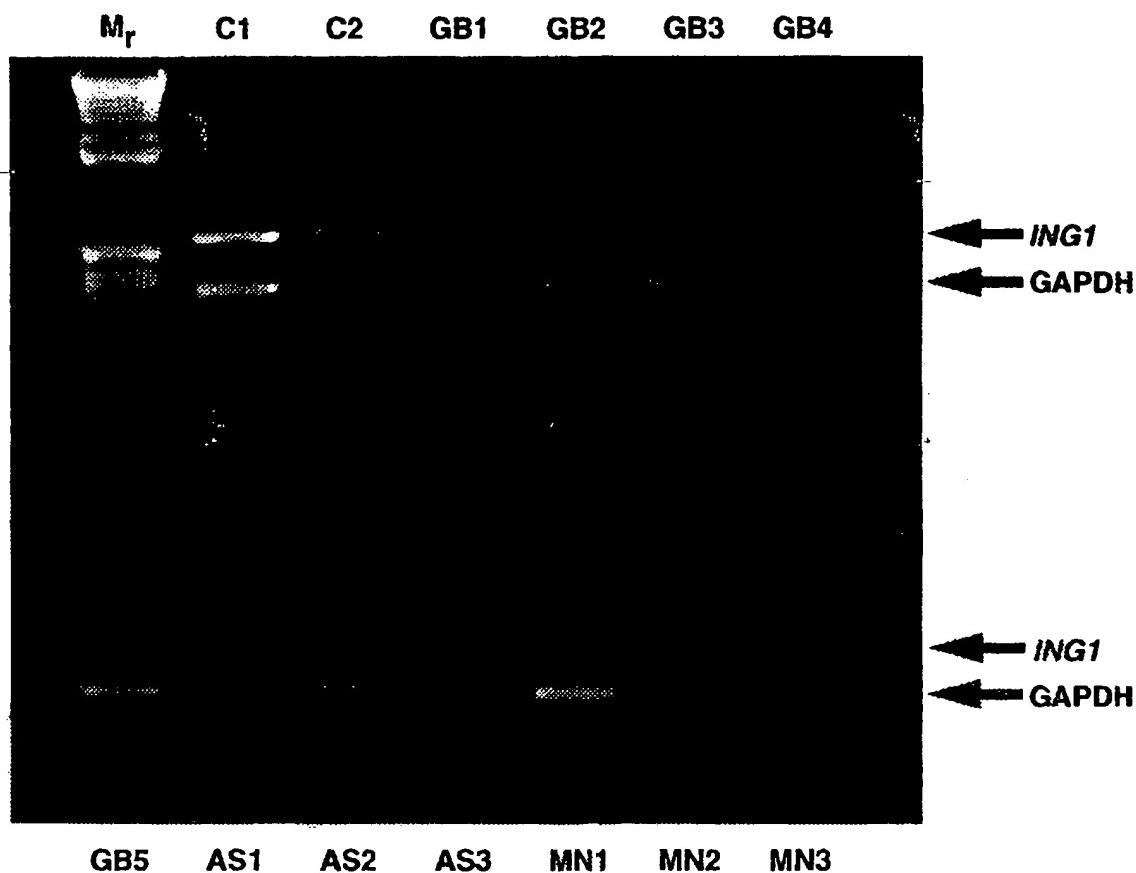
restriction analysis

**FIGURE 6c**

PCR analysis



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Loss of *ING1* expression in multiple forms of Brain Cancers**FIGURE 7****SUBSTITUTE SHEET (RULE 26)**

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Expression of *ING1*/p33^{ING1} in
low & high passage primary HDFs

mRNA levels by RT-PCR

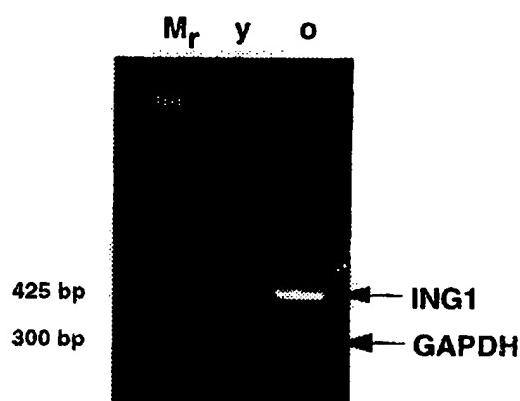


FIGURE 8a

Protein levels by western blot

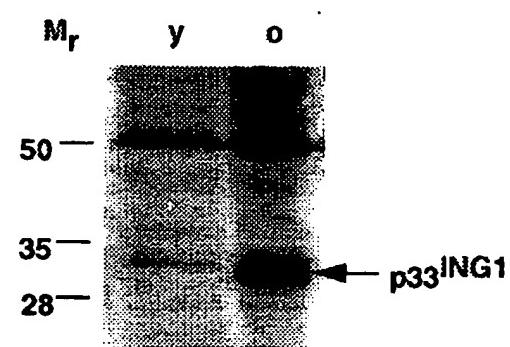
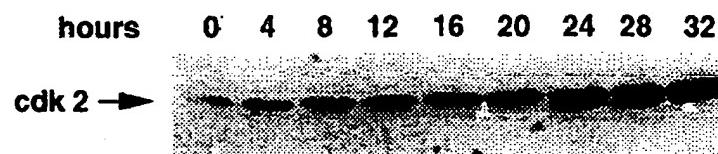
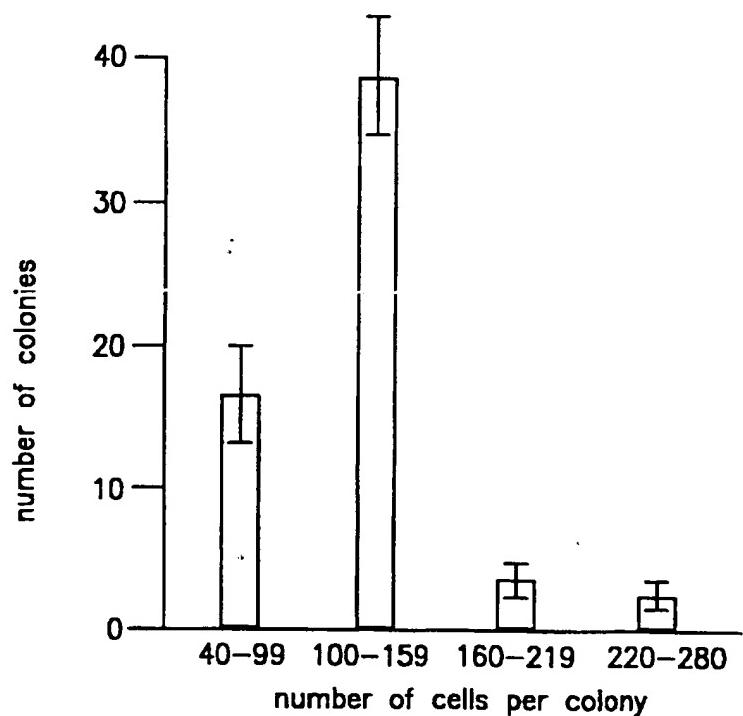


FIGURE 8b

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Expression of p33^{ING1} through the cell cycle**FIGURE 9a****FIGURE 9b****FIGURE 9c**

	0	4	8	12	16	20	24	28	32
G0/G1	87.0	87.3	88.0	89.2	72.2	61.7	50.7	50.8	58.9
S	6.2	4.7	3.1	5.0	17.7	30.4	28.5	22.3	19.1
G2/M	6.8	8.0	8.9	5.8	10.1	8.0	20.8	21.9	22.0

9/9**FIGURE 10**

INTERNATIONAL SEARCH REPORT

Int'l Application No.
PCT/CA 96/00819

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C07K16/18 C12Q1/68 A61K48/00
A61K38/17 C12N15/11 C12N15/86 G01N33/574 G01N33/68
C12N5/10 C12N15/62 C12N15/89

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL ENTRY HS12525 Accession number T53125, 27 February 1995 HILLIER ET AL.: "WasU-Merck EST Project" XP002028241 see abstract & UNPUBLISHED, ---	1-3, 11-13
A	WO 95 25429 A (MYRIAD GENETICS INC ;KAMB ALEXANDER (US)) 28 September 1995 see page 9, line 25 - page 11, line 3 see page 13, line 17 - page 19, line 2 see page 35, line 1 - page 44, line 25 ---	5-10, 20-33
A	SCIENCE, vol. 254, 22 November 1991, pages 1138-1146, XP002028240 WEINBERG R. A.: "Tumor Suppressor Genes" ---	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

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Date of the actual completion of the international search 27 March 1997	Date of mailing of the international search report 08.04.97
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Fax (+31-70) 340-3016

Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

In' tional Application No
PCT/CA 96/00819

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 00329 A (BIOGEN INC ;UNIV CALIFORNIA (US)) 9 January 1992 see abstract ----	5-10,17, 20-25, 30,33
A	WO 90 02569 A (INT GENETIC ENG) 22 March 1990 see page 40, paragraph 2A - page 41 see page 53, paragraph 7A - page 54 ----	28,31
T	NATURE GENETICS, vol. 14, no. 4, December 1996, pages 415-420, XP000647739 GARKAVTSEV ET AL.: "Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation" see the whole document -----	1-33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 96/00819

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
see continuation-sheet
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 96/00819

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 9,10,21-24,28-31, and partially claims 5-8 and 25-27 as far as concerning an in-vivo method, are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 96/00819

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9525429 A	28-09-95	AU 2186395 A CA 2162147 A CN 1128935 A EP 0708592 A FI 955415 A JP 8510651 T NO 954494 A AU 2378995 A CA 2162150 A CN 1128049 A EP 0702730 A FI 955416 A JP 8510391 T NO 954495 A WO 9525813 A		09-10-95 28-09-95 14-08-96 01-05-96 09-01-96 12-11-96 17-01-96 09-10-95 28-09-95 31-07-96 27-03-96 09-01-96 05-11-96 17-01-96 28-09-95
WO 9200329 A	09-01-92	AU 2716795 A AU 8228891 A CA 2086264 A EP 0536299 A JP 6501456 T		15-02-96 23-01-92 28-12-91 14-04-93 17-02-94
WO 9002569 A	22-03-90	AU 643189 B AU 4402189 A EP 0364096 A US 5576184 A AU 627591 B AU 5754790 A CA 2019323 A EP 0404003 A JP 3280884 A		11-11-93 02-04-90 18-04-90 19-11-96 27-08-92 20-12-90 19-12-90 27-12-90 11-12-91